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1. Journal of Immunology:
   1998, Vol. 161, pp. 4467-4471
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2. Nature:
   1998, Vol. 393, pp. 478-480
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3. Journal of Experimental Medicine:
   1997, Vol. 186, pp. 239-245
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4. Science, 1991, Vol. 252, pp. 1424-1427
5. Trends in Immunology, 2001, Vol. 7, pp. 394-400
6. Clin Biochem, 1998, Vol. 31, pp. 335-340

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Help for cytotoxic-T-cell responses is mediated by CD40 signalling

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Cytotoxic T lymphocytes (CTLs) which carry the CD8 antigen recognize antigens that are presented on target cells by the class I major histocompatibility complex. CTLs are responsible for the killing of antigen-bearing target cells, such as virus-infected cells. Although CTL effectors can act alone when killing target cells, their differentiation from naive CD8-positive T cells is often dependent on 'help' from CD4-positive helper T (T_H) cells[1–4]. Furthermore, for effective CTL priming, this help must be provided in a cognate manner, such that both the T_H cell and the CTL recognize antigen on the same antigen-presenting cell[2,4]. One explanation for this requirement is that T_H cells are needed to convert the antigen-presenting cell into a cell that is fully competent to prime CTL[5]. Here we show that signalling through CD40 on the antigen-presenting cells can replace the requirement for T_H cells, indicating that T-cell 'help', at least for generation of CTLs by cross-priming, is mediated by signalling through CD40 on the antigen-presenting cell.

CD8-positive CTLs are responsible for the lysis of antigen-bearing target cells. These CTLs recognize peptide antigens presented by class I molecules encoded within the major histocompatibility complex (MHC). Generation of effective CTL responses often requires help from a second subset of T lymphocytes, the CD4-positive helper T (T_H) cells[1–4], but this is not always the case[6,7]. In response to the soluble protein ovalbumin (OVA), both T_H-cell-dependent and T_H-cell-independent CTL immunity can be induced[4,8]. When the K^b-restricted OVA peptide determinant spanning residues λ57 to 264 (OVAp) was emulsified in complete Freund's adjuvant (CFA) and injected subcutaneously, CTLs could be generated in mice lacking CD4-positive T cells (Fig. 1a)[4]. In contrast, priming by intravenous injection of irradiated spleen cells loaded with OVA by osmotic shock (OVA-loaded spleen cells) required the presence of CD4-positive T cells (Fig. 1b). This latter form of immunization occurs by cross-priming[4,9], requiring representation of antigen by host bone-marrow-derived antigen-presenting cells (APCs). Dissection of the cellular interactions involved in this response[4] revealed that, like the induction of CTLs that are specific for the Qa1 antigen[1], the T_H and CTL populations must recognize OVA on the same APC for effective CTL priming. This could be explained in two ways: either the T_H cells need to closely associate with the CTL to deliver short-range signals such as interleukin(IL)-2 (ref. 1), or they are required to modify the APC, converting it into a stimulatory cell for CTL priming[5].

There is evidence that CD40 and CD40 ligand (CD154) are important in both the humoral and cellular immune responses (reviewed in refs 10, 11). These molecules have been implicated in the generation of CTL responses to adenovirus[12] and in the estab-

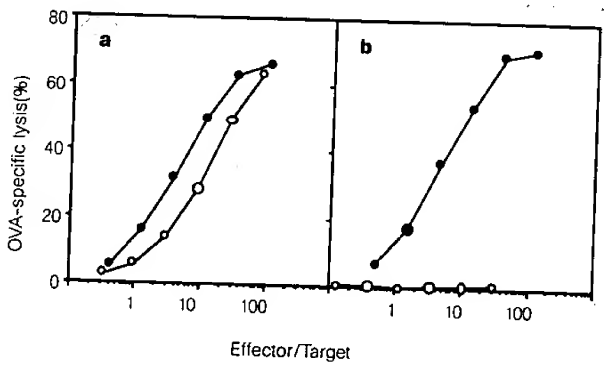

Figure 1 OVA-specific CTLs can be generated in CD4-independent and CD4-dependent ways. Normal B6 mice (filled circles) or B6 mice depleted of CD4-positive T cells by twice-weekly intraperitoneal injection of 100 μl GK1.5 ascites[4] (open circles) were injected either **a**, subcutaneously with 20 μg OVAp in 200 μl CFA, or **b**, intravenously with irradiated B6 OVA-loaded spleen cells as described[4]. After 8 days, spleen cells from each mouse were restimulated for 6 days *in vitro* as described[4]. On the day of assay, effector cells were examined for their ability to lyse ^{51}Cr-labelled EL4 targets that were or were not pulsed with OVAp. Non-specific EL4 lysis was <10%.

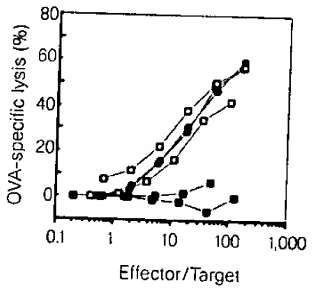

Figure 2 Treatment with a monoclonal antibody against CD40 replaces CD4-positive T-cell help in generating OVA-specific CTLs. Normal B6 mice (circles) and H-2A^b-deficient B6 mice (squares), were injected intravenously with irradiated, OVA-loaded bm1 spleen cells[4]. They were then left untreated (circles), or were injected intravenously daily for 4 days with either 0.1 mg of a CD40-specific monoclonal antibody, FGK45 (ref. 17) (open squares) or an IgG2a isotype control, KT50, specific for Vα8 (filled squares). Eight days after priming, the spleen cells from each mouse were restimulated *in vitro* for six days. We then examined their ability to lyse ^{51}Cr-labelled EL4 targets that were or were not pulsed with OVAp. Nonspecific EL4 lysis was <7%.

lishment of CTL memory to lymphocytic choriomeningitis virus[13]. CD154-deficient mice are unresponsive to adenovirus in that they do not generate either antibody or CTLs[14], unless co-injected with a CD40-stimulating monoclonal antibody. However, it was not determined which cellular population was normally responsible for supplying CD154 for CTL induction; CD4-positive T_H cells, CD8-positive CTLs, or some other cell type. We reasoned, however, that as this ligand is mainly expressed by activated T_H cells[15,16], CD154 signalling to CD40 may represent the 'help' provided by these cells during CTL generation. To test this, we determined whether a CD40 stimulus could substitute for T_H cells in the CTL response to OVA-loaded spleen cells. Mice lacking the MHC locus H–2A^b, which do not express MHC class II molecules and therefore lack class II-restricted CD4-positive T_H cells, were primed with OVA-loaded spleen cells in the presence of either a CD40-stimulating monoclonal antibody, FGK45 (ref. 17), or an isotype-control antibody. We then examined OVA-specific CTL generation (Fig. 2). We used spleen cells from bm1 mice as the OVA-loaded priming source to ensure that stimulation could only occur through cross-priming on host APCs, as the H–2^{bm1} haplotype cannot directly present OVA to CD8-positive T cells. In this case, strong CTL responses were induced in the presence, but not the absence, of the CD40-specific monoclonal antibody. There was no CTL priming when mice were given FGK45 in the absence of OVA-loaded spleen cells (data not shown).

To exclude the possibility that the few unusual CD4-positive T cells in H–2A^b-deficient mice could substitute for T_H cells when a CD40 stimulus was provided, we performed similar experiments in thymectomized B6 mice that were depleted of CD4-positive T cells by administration of monoclonal antibody (Fig. 3). This showed that, even when CD4-positive T cells were depleted by antibody, mice could be primed only in the presence of a CD40-stimulating monoclonal antibody. These data indicate that a CD40 stimulus can substitute for T_H cells, suggesting that help is normally mediated through CD40 signalling following engagement by CD154 expressed on T_H cells.

An alternative explanation, however, is that signalling by the CD40-specific monoclonal antibody substituted for a completely different signal supplied by T_H cells. To exclude this possibility, we examined OVA-specific CTL responses in CD40-deficient or CD154-deficient mice. If CD40 signalling is normally involved in this form of priming, these mice should not generate OVA-specific CTL responses. CTL responses were indeed poor in both CD154-deficient mice (Fig. 4a) and CD40-deficient mice (Fig. 4b). In one of four experiments, using CD40-deficient mice, we detected relatively efficient CTL responses (data not shown). As we performed this experiment soon after introducing the CD40-deficient mice into our colony, we suspect that subclinical infections provided inflammatory signals that substituted for the CD154 signal of T_H cells, in much the same way as CFA replaced the need for T_H cells in the generation of OVA-specific CTLs[4,8] (Fig. 1a). The general lack of CTL responses in mice lacking either CD40 or CD154 support a role for CD40 signalling in the cross-priming of OVA-specific CTLs.

Priming of CTLs by OVA-loaded spleen cells occurs through antigen presentation by host APCs. This priming requires help mediated by a T_H-cell interaction with the same APCs as seen by the CTLs[4]. The requirement for CD40 and CD154 in this response (Fig. 4), and the ability of a CD40-specific monoclonal antibody to replace T_H cells (Figs 2, 3), indicates that CD4-positive T-cell help mainly supplies CD154 for CD40 signalling. As CD40 is expressed by APCs such as dendritic cells (reviewed in ref. 10), and as signalling through CD40 is important for maturation of several antigen-presenting functions[14,18–25], the cross-priming APC probably represents the target cell that is stimulated through CD40. B cells, which also express CD40, are unlikely to be involved in this response, as presentation of antigen by this population is tolerogenic for naive CD8-positive T cells, even in the presence of T_H cells[26]. A small proportion of CD8-positive T cells has been reported to express CD40 (ref. 27), so a direct interaction between CD154 on the T_H cell and CD40 on the CTL may occur. This would, however, require interaction between these cells while associated on the APC, as murine CTLs do not express MHC class II molecules.

How signalling through CD40 enables the cross-priming APC effectively to stimulate CTLs is unclear, although CD40 signalling may lead to the upregulation of co-stimulatory molecules such as B7 (refs 14, 19), CD44H (ref. 20) and ICAM-1 (ref. 21) on various cell types, and to the secretion of cytokines, such as tumour-necrosis factor-α, IL-1β and IL-12 (refs 10, 19,22–25). Whether these or other events form the critical downstream helper effects provided by T_H cells is unknown. Although CD40–CD154 interactions are important for the induction of CTL immunity to adenovirus[12], they are not essential for induction of CTL responses to several other viruses[13,28]. We suggest that, like CFA, which allows the induction of T_H-cell-independent CTL responses to OVA, some viruses may

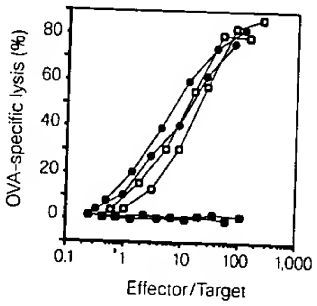

Figure 3 A CD40-specific monoclonal antibody can replace CD4-positive T-cell help in the induction of OVA-specific CTLs. Thymectomized B6 mice were depleted of CD4-positive T cells by twice-weekly intraperitoneal injection of GK1.5 acsites[4] (squares) or were left untreated (circles). All mice were then immunized with 25 × 10^6 OVA-loaded B6 spleen cells. CD4-depleted mice were then either treated with 0.1 mg anti-CD40 antibody intravenously daily for 7 days (open squares), or left untreated (filled squares). Spleen cells were then cultured *in vitro* for 6 days with 10^7 irradiated E.G7 cells. The resulting effector cells were then included in a chromium-release assay using EL4 target cells that were or were not pulsed with OVAp. Nonspecific EL4 lysis was <5%.

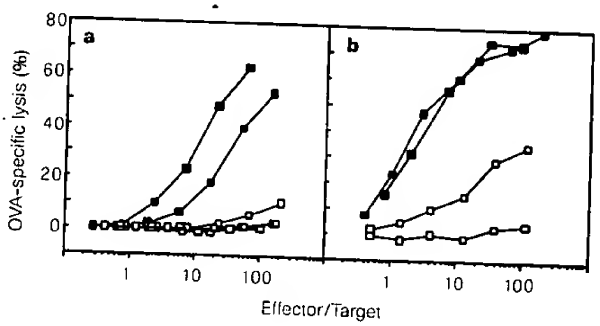

Figure 4 The role of CD154 and CD40 in the generation of OVA-specific CTLs by cross-priming. **a**, We primed four CD154-deficient mice[29] (open squares) and two normal B6 mice (filled squares) with bm1 OVA-loaded spleen cells, and then examined for CTL generation. We performed this experiment twice, with similar results. **b**, We immunized two CD40-deficient[30] mice (open squares) and two normal B6 mice (filled squares) intravenously with bm1 OVA-loaded spleen cells, and then examined for CTL generation. We performed this experiment four times, with three experiments giving similar results. Nonspecific EL4 lysis was <36%.

provide inflammatory signals that activate APCs directly, circumventing their need for activation through CD40. Perhaps the requirement for CD40 signalling depends on whether the professional APC is directly infected or presents antigen indirectly by cross-presentation. Alternatively, there may be other inherent properties of some viruses that allow CD40-independent activation of APCs.

The ability to replace T_H cells with an antibody against CD40 may provide an opportunity for vaccination under circumstances that were previously difficult, either because of a lack of available class II-restricted antigenic determinants (as may occur with some tumour antigens) or because of a deficiency in CD4-positive T_H cells (as occurs in AIDS patients). Further dissection of the molecular interactions involved in the generation of immunogenic CTL responses may help in vaccine design and function. □

Methods

Generation of CTL responses. CTL responses to OVA were generated as described[4]. Briefly, mice were primed with 25×10^6 OVA-loaded spleen cells. Seven to eight days later, we restimulated spleen cells from primed mice *in vitro* for six days with irradiated (1,500 centiGray) OVA-loaded B6 spleen cells or irradiated (20,000 centiGray) E.G7 tumour cells. We then examined the ability of the primed spleen cells to lyse ^{51}Cr-labelled EL4 target cells, pulsed or unpulsed with OVAp as described[4]. The percentage of OVA-specific lysis was then calculated as the percentage of OVAp-pulsed EL4 lysis minus the percentage of EL4 lysis. To generate CTLs in CD154-deficient[29] or CD40-deficient[30] mice, we modified the *in vitro* culture conditions slightly to avoid responses to mouse strain 129 minor histocompatibility antigens. Seven days after immunization with OVA-loaded bm1 spleen cells, we removed 'responder' spleens and irradiated half the cells from each spleen (1,500 centiGray). We then loaded these irradiated cells with OVA with OVA by osmotic shock and used these cells as *in vitro* stimulators for the remaining splenocytes. We combined unirradiated 'responder' spleen cells (3×10^6 cells ml^{-1}) with irradiated OVA-loaded cells from the same animal (3×10^6 cells ml^{-1}) and cultured them *in vitro* for six days in several 2-ml cultures. On day 6, we pooled effector cells from each mouse and used them in a chromium-release assay as described[4].

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T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions

Stephen P. Schoenberger*†‡, Rene E. M. Toes*†, Ellen I. H. van der Voort*, Rienk Offringa* & Cornelis J. M. Melief*

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† These authors contributed equally to this work.

Although *in vivo* priming of CD8$^+$ cytotoxic T lymphocytes (CTLs) generally requires the participation of CD4$^+$ T-helper lymphocytes[1,2], the nature of the 'help' provided to CTLs is unknown[3]. One widely held view is that help for CTLs is mediated by cytokines produced by T-helper cells activated in proximity to the CTL precursor at the surface of an antigen-presenting cell (APC)[4]. An alternative theory is that, rather than being directly supplied to the CTL by the helper cell, help is delivered through activation of the APC, which can then prime the CTL directly[5]. CD40 and its ligand, CD40L, may activate the APC to allow CTL priming. CD40L is expressed on the surface of activated CD4$^+$ T-helper cells and is involved in their activation and in the development of their effector functions[6,7]. Ligation of CD40 on the surface of APCs such as dendritic cells, macrophages and B cells greatly increases their antigen-presentation and co-stimulatory capacity[8–11]. Here we report that signalling through CD40 can replace CD4$^+$ T-helper cells in priming of helper-dependent CD8$^+$ CTL responses. Blockade of CD40L inhibits CTL priming; this inhibition is overcome by signalling through CD40. CD40–CD40L interactions are therefore vital in the delivery of T-cell help for CTL priming.

The dependence on 'help' mediated by CD4$^+$ T cells for the priming of antigen-specific CD8$^+$ CTL responses has been well documented[1–4]. Despite this, the mechanism through which help is

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provide inflammatory signals that activate APCs directly, circumventing their need for activation through CD40. Perhaps the requirement for CD40 signalling depends on whether the professional APC is directly infected or presents antigen indirectly by cross-presentation. Alternatively, there may be other, inherent properties of some viruses that allow CD40-independent activation of APCs.

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T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions

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Although *in vivo* priming of CD8$^+$ cytotoxic T lymphocytes (CTLs) generally requires the participation of CD4$^+$ T-helper lymphocytes[1,2], the nature of the 'help' provided to CTLs is unknown[3]. One widely held view is that help for CTLs is mediated by cytokines produced by T-helper cells activated in proximity to the CTL precursor at the surface of an antigen-presenting cell (APC)[4]. An alternative theory is that, rather than being directly supplied to the CTL by the helper cell, help is delivered through activation of the APC, which can then prime the CTL directly[5]. CD40 and its ligand, CD40L, may activate the APC to allow CTL priming. CD40L is expressed on the surface of activated CD4$^+$ T-helper cells and is involved in their activation and in the development of their effector functions[6,7]. Ligation of CD40 on the surface of APCs such as dendritic cells, macrophages and B cells greatly increases their antigen-presentation and co-stimulatory capacity[8–11]. Here we report that signalling through CD40 can replace CD4$^+$ T-helper cells in priming of helper-dependent CD8$^+$ CTL responses. Blockade of CD40L inhibits CTL priming; this inhibition is overcome by signalling through CD40. CD40–CD40L interactions are therefore vital in the delivery of T-cell help for CTL priming.

The dependence on 'help' mediated by CD4$^+$ T cells for the priming of antigen-specific CD8$^+$ CTL responses has been well documented[1–4]. Despite this, the mechanism through which help is

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provided to CTLs has remained unknown. We used a well characterized model system to probe the mechanism of T-cell help for the primary activation of $CD8^+$ CTL responses *in vivo*. C57BL/6 (B6; with the major histocompatibility complex (MHC) $H-2^b$) mice immunized with allogeneic BALB/c ($H-2^d$) mouse embryo cells (MECs) expressing the human adenovirus type 5 early region 1 (Ad5E1–BALB/c MECs) generated strong CTL responses against an $H-2D^b$-restricted epitope of the adenovirus E1B protein ($E1B_{192-200}$) (Fig. 1a)[12]. As the allogeneic $H-2^d$ MHCs expressed by the immunizing MECs cannot prime $H-2^b$-restricted host CTLs[12], generation of E1B-specific CTLs must require cross-priming, that is, the uptake and $H-2^b$-restricted re-presentation of antigen by host APCs[13,14]. Cross-priming of E1B-specific CTLs is strictly helper-dependent (Fig. 1b), as mice depleted of $CD4^+$ T-helper (T_H) cells before immunization no longer mounted an E1B-specific CTL response.

Studies of the requirement for T-cell help in cross-priming of CTLs have shown that both T_H cells and CTLs must recognize antigens presented on the same APC[3,4]. Although this could be explained by a proximity requirement for the efficient delivery from T_H cells of soluble factors such as interleukin (IL)-2, another possibility is that a cognate interaction between T_H cells and APCs is required to convert the APC to a state that is capable of directly priming naive CTLs[5]. CD40 and CD40L (CD154) may mediate the activation of APCs by T_H cells. CD40 (expressed on APCs) and CD40L (expressed on activated T_H cells) are important in both humoral and cellular immune responses (reviewed in refs 15, 16). A failure to properly activate APCs could explain many of the immune defects of CD40L-deficient mice, such as the inability to prime antigen-specific T cells, to resist infection with *Leishmania*, or to reject tumours following vaccination with tumour cells[6,17,18].

To investigate whether signalling through CD40 can replace $CD4^+$ T_H cells in CTL priming, we depleted mice of $CD4^+$ cells *in vivo* before immunization with Ad5E1–BALB/c MECs. One day after immunization, the mice received a single injection of an anti-CD40 activating antibody, FGK45 (ref. 19), or of an isotype-matched control antibody. Administration of FGK45 to CD4-depleted, immunized mice resulted in the efficient restoration of E1B-specific CTL responses (Fig. 2a) whereas treatment with the control antibody did not (Fig. 2b). Priming of E1B-specific CTLs was not detected in naive mice treated with FGK45 alone (not shown). To address the possibility that the effect of FGK45 was mediated through remaining $CD4^+$ cells that were not depleted by treatment with the anti-CD4 antibody, we immunized B6 $I-A^b$ knockout mice, which lack functional MHC class II-restricted $CD4^+$ peripheral T cells[20], with the Ad5E1–BALB/c MECs. The response to immunization in these mice mirrors that seen in the CD4-depleted mice, in that E1B-specific CTLs were detectable only in mice receiving the CD40-activating antibody (Fig. 2c), and not in those receiving the control antibody (Fig. 2d). We studied whether the requirement for anti-CD40 antibodies in priming of CTLs in CD4-depleted mice could be replaced by administration of bacterial lipopolysaccharide (LPS) (50 μg intravenous), a potent inducer of proinflammatory cytokines, or IL-2 (1×10^5 Cetus units in incomplete Freund's adjuvant, subcutaneous) following immunization with Ad5E1–BALB/c MECs. Whereas CD4-depleted mice treated with FGK45 exhibited strong E1B-specific CTL activity, neither LPS nor IL-2 treatment resulted in detectable CTL priming (not shown). We also observed no binding of FGK45 to $CD8^+$ CTLs from either naive or immunized mice, although significant binding to surface-immunoglobulin-positive B cells was consistently observed (not shown). These results indicate that activation through CD40 can bypass the requirement for $CD4^+$ T-helper cells in the cross-priming of E1B-specific CTLs.

Ligation of CD40 on B cells upregulates their costimulatory activity[11,21], suggesting a role for these cells in the restoration of CTL priming by treatment with anti-CD40 antibodies. To address this question, we immunized B6 μMT mice, which lack mature B cells[22], with the allogeneic Ad5E1–BALB/c MECs. Cross-priming of E1B-specific CTLs did not require mature B cells (Fig. 3a). However, when depleted of $CD4^+$ cells, the B-cell-deficient mice did not generate an E1B-specific CTL response (Fig. 3b). Activation through CD40 with FGK45 completely restored the capacity of the CD4-depleted μMt mice to prime E1B-specific CTLs (Fig. 3c). Thus B cells are not required as APCs or accessory cells for cross-priming in our system, nor are they required for the CD40-mediated restoration of cross-priming of CTLs in the absence of $CD4^+$ T-helper cells.

Our results thus far are consistent with the idea that FGK45-mediated restoration of CTL priming operates through activation

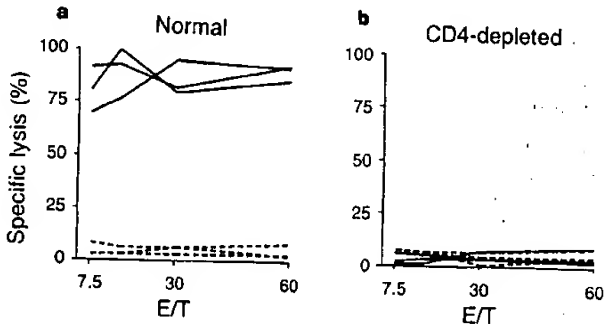

Figure 1 Cross-priming of E1B-specific CTLs requires $CD4^+$ T-helper cells. Splenocytes from **a**, normal, or **b**, CD4-depleted B6 mice immunized with Ad5E1-BALB/c MECs were tested at various effector/target (E/T) ratios for lysis of syngeneic MEC target cells loaded with the $E1B_{192-200}$ peptide (solid lines) or a D^b-restricted control peptide, HPV-16 $E7_{49-57}$ (dashed lines). Each line represents one mouse. Data shown represent one experiment of three performed with similar results.

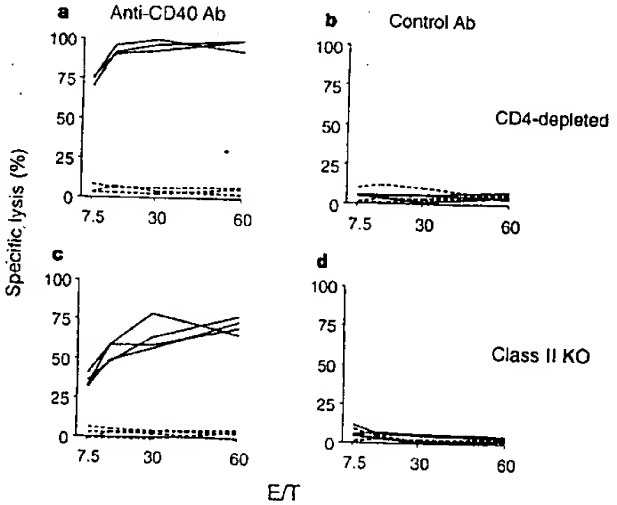

Figure 2 CD40 activation replaces $CD4^+$ helper T cells. Splenocytes from **a**, **b**, CD4-depleted, or **c**, **d**, class-II-deficient $I-A^b$-knockout (KO) B6 mice were immunized with Ad5E1-BALB/c MECs and treated with either the CD40-activating antibody (Ab) FGK45 (**a**, **c**), or an isotype control antibody (**b**, **d**). These splenocytes were tested for E1B-specific CTL activity on syngeneic MEC target cells loaded with either the $E1B_{192-200}$ peptide (solid lines) or the HPV $E7_{49-57}$ control peptide (dashed lines). Each line represents a single mouse. Data depicted are derived from two independent experiments. E/T, effector/target ratio.

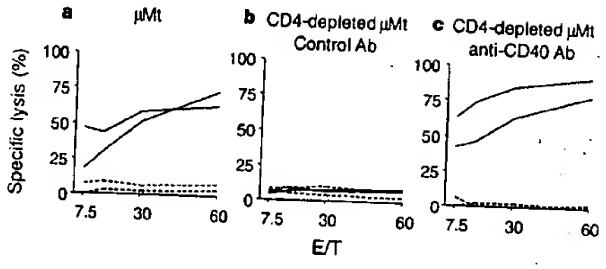

Figure 3 B cells are not essential as cross-priming APCs or for anti-CD40-mediated restoration of cross-priming. Splenocytes were taken from **a**, untreated, or **b**, **c**, CD4-depleted B-cell-deficient μMt B6 mice which were immunized with Ad5E1-BALB/c MECs and which received either an isotype control antibody (**b**) or the CD40-activating antibody FGK45 (**c**). These splenocytes were tested for E1B-specific CTL activity on syngeneic MEC target cells loaded with either the $E1B_{192-200}$ peptide (solid lines) or the HPV $E7_{49-57}$ control peptide (dashed lines). Each line represents one mouse. Data shown represent one experiment of two performed with similar results. E/T, effector/target ratio μMT, B-cell-deficient mice.

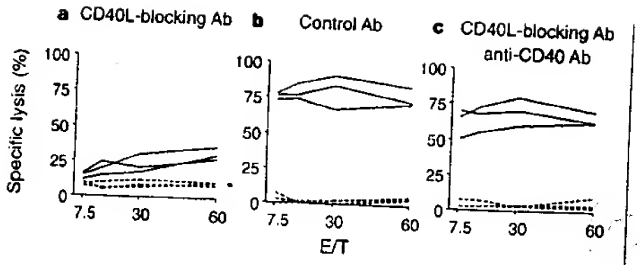

Figure 4 CD40L blockade prevents cross-priming of E1B-specific CTLs. Splenocytes were taken from **a**, **b**, B6 mice immunized with Ad5E1-BALB/c MECs and treated with **a**, the CD40L-blocking antibody MR1, or **b**, hamster IgG control antibodies, or **c**, from mice treated with the CD40L-blocking antibody that received the CD40-activating antibody FGK45 24 h after immunization. These splenocytes were tested for E1B-specific CTL activity on syngeneic MEC target cells loaded with the $E1B_{192-200}$ peptide (solid lines) or the HPV $E7_{49-57}$ control peptide (dashed lines). Each line represents one mouse. Data shown represent one experiment of two performed with similar results. E/T, effector/target ratio.

of CD40 on a non-B-cell APC, most likely of the dendritic cell/macrophage lineage. If this represents a physiological pathway used by $CD4^+$ T cells to help CTLs, blocking the ability of the $CD4^+$ T cells to interact with APC through CD40L–CD40 interactions would be expected to diminish priming of E1B-specific CTL responses in normal mice. We immunized B6 mice with Ad5E1-BALB/c MECs and then treated them with either a CD40L-blocking antibody, MR1 (ref. 23), or control antibodies. Blockade of CD40L results in drastically reduced E1B-specific CTL responses (Fig. 4a) compared with the efficient CTL priming seen in mice receiving the control antibodies (Fig. 4b). The priming defect induced by CD40L blockade was fully restored following CD40 signalling by FGK45 (Fig. 4c). Thus the defect induced by CD40L blockade lies in the failure of T_H cells to transmit, rather than to receive, CD40L-mediated signals.

Our results reaffirm the notion that priming of virgin CTLs *in vivo* requires the interaction of three distinct cell types: the antigen-specific $CD4^+$ and $CD8^+$ T cells and the antigen-bearing APC. However, in contrast to the model proposed by Keene and Forman[4], our results indicate that, rather than directly stimulating the CTL, the contribution of $CD4^+$ T_H cells involves the activation of professional (non-B-cell) APCs through CD40–CD40L interactions. These data support the model of T-cell help for CTLs that was first proposed by Guerder and Matzinger[5] and identify CD40–CD40L interactions as mediating the activation of APC by T_H cells. This mechanism of T-cell help allows the interaction of three cell types to be accomplished through sequential, two-cell interactions, the first involving T_H-mediated activation of APCs, which are then able to prime CTLs directly.

The recovery of CTL responses by treatment with an activating antibody against CD40 has been seen by infecting CD40L-deficient mice with a recombinant adenovirus[24]. However, in this study the role of T_H cells in CTL priming was not addressed, as the CD40L-deficient mice used were not depleted of $CD4^+$ T cells. Here we have shown that anti-CD40 antibodies allow the priming of helper-dependent CTLs in the physical, or functional, absence of $CD4^+$ cells.

Although the identity of the host APCs involved in cross-priming remains unknown, our results rule out a requirement for B cells in our system, either as APCs or as sources of nonspecific cytokine/costimulatory signals in response to CD40 activation. Thus, the effect of anti-CD40 antibodies may be mediated through the activation of a $CD40^+$ non-B-cell APC. Among the other CD40-expressing APCs, dendritic cells are those most capable of priming T cells[25]. It is therefore likely that the mechanism by which CD40 signalling allows helper-independent CTL priming involves one or more of the alterations that are induced by ligation of CD40 on dendritic cells, such as upregulation of surface CD80/CD86 levels or the production of IL-12 (refs 8, 9). Conversely, the mechanism of CD40-mediated CTL priming may involve the interruption of negative signals transmitted by the APC.

Our results provide new insights into the nature of helper-dependent CTL responses and suggest methods for their manipulation. Vaccination strategies based on the activation of APCs through CD40 may be useful in eliciting CTL responses in situations where the number or activity of $CD4^+$ T_H cells is limiting, such as occurs in AIDS patients. These findings are also relevant to tumour immunology, as they indicate that a lack of tumour-specific T_H-cell determinants may prevent efficient priming of tumour-specific CTLs. By combining active vaccination with CD40 signalling *in vivo*, however, a more effective anti-tumour immune response may be realized. CD40 signalling may also be useful in achieving the full CTL-stimulatory potential of dendritic-cell-based vaccines prepared *in vitro*[26]. Finally, our data contribute to knowledge of the emerging role of CD40–CD40L interactions as key elements in the regulation of both T- and B-cell immune responses. The linkage of CTL priming to T_H-dependent APC activation imposes an important regulatory control point in the induction of immune responses against self and non-self antigens. Cross-presentation of ovalbumin (OVA) by bone-marrow-derived APCs has been shown to lead to deletion of OVA-specific CTLs when OVA is expressed as a self antigen on healthy tissues[27]. This contrasts with the activation of OVA-specific CTLs by cross-priming APCs, which occurs when OVA-loaded cells are used for immunization[4]. Our results indicate that the activation state of the APC, which depends on a cognate interaction with T_H cells mediated by CD40–CD40L interactions, may be important in orchestrating these different outcomes in the response to the same antigen. A new understanding of the importance of CD40–CD40L interactions for CTL priming and tolerance should allow the development of new methods for both vaccination and tolerance induction. □

Methods

Mice, cell lines, and immunizations. 6–12-week-old male B6 ($H-2^b$) mice used in this study were obtained from the Netherlands Cancer Institute (Amsterdam). B6I-A^b-knockout and IgM-knockout (μMT) mice were obtained from P. Matzinger. The generation of Ad5E1-BALB/c ($H-2^d$) MECs and Ad5E1-B6 MECs has been described[13,28]. All *in vitro* cultures were grown in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Paisley)

supplemented with 8% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, glutamine and penicillin (culture medium). B6 mice were immunized with 1×10^7 irradiated (100 Gy) Ad5E1–BALB/c MECs in 0.2 ml PBS injected subcutaneously in the flank. Two weeks later, spleens were removed and single-cell suspensions prepared by mechanical disruption. 5×10^6 unseparated splenocytes were restimulated with 5×10^5 irradiated Ad5E1–B6 MECs in 2-ml cultures in 24-well plates. No additional growth factors or cytokines were added to these cultures.

Cytotoxicity assays. Following a 6-day culture, viable lymphocytes were isolated from splenocyte cultures and used as effectors in europium-release cytotoxicity assays as described[12]. The mean percentage specific lysis of triplicate cultures was calculated as follows: specific lysis = [(c.p.m. experimental release − c.p.m. spontaneous release/(c.p.m. maximum release − c.p.m. spontaneous release)] × 100. Spontaneous release was always <20%. Peptides were synthesized using solid-phase chemistry on a Abimed AMS 422 peptide synthesizer.

***In vivo* antibody treatment.** Mice were depleted of $CD4^+$ cells by three consecutive intraperitoneal (i.p.) injections of 100 μg of the anti-CD4 antibody GK1.5 (ref. 29) given every other day before immunization. Depletion was confirmed by fluorescence-activated cell sorting analysis using the RM4-4 antibody (Pharmingen), which does not compete with GK1.5 for binding to CD4. Depleted populations contained <0.25% CD4 cells. Depletion of CD4 cells was maintained by administration of 100 μg GK1.5 every 5–7 days until spleens were removed two weeks after immunization.

For CD40 activation, mice received 100 μg purified CD40-activating antibody FGK45 or a rat IgG2a control antibody (Biosource, Belgium) given intravenously in 0.2 ml PBS 24 h after immunization with Ad5E1–BALB/c MECs.

To block CD40L, mice received 250 μg HPLC-purified CD40L-blocking antibody MR1 (ref. 23) or hamster IgG control antibodies in 0.2 ml PBS given intraperitoneally every other day for 11 days beginning on the day of immunization with Ad5E1–BALB/c MECs.

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Role of Mxi1 in ageing organ systems and the regulation of normal and neoplastic growth

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Mxi1 belongs to the Mad (Mxi1) family of proteins, which function as potent antagonists of Myc oncoproteins[1–4]. This antagonism relates partly to their ability to compete with Myc for the protein Max and for consensus DNA binding sites and to recruit transcriptional co-repressors[4–6]. Mad(Mxi1) proteins have been suggested to be essential in cellular growth control and/or in the induction and maintenance of the differentiated state[6,7]. Consistent with these roles, *mxi1* may be the tumour-suppressor gene that resides at region 24–26 of the long arm of chromosome 10. This region is a cancer hotspot, and mutations here may be involved in several cancers, including prostate adenocarcinoma[8–10]. Here we show that mice lacking Mxi1 exhibit progressive, multisystem abnormalities. These mice also show increased susceptibility to tumorigenesis either following carcinogen treatment or when also deficient in Ink4a. This cancer-prone phenotype may correlate with the enhanced ability of several *mxi1*-deficient cell types, including prostatic epithelium, to proliferate. Our results show that Mxi1 is involved in the homeostasis of differentiated organ systems, acts as a tumour suppressor *in vivo*, and engages the Myc network in a functionally relevant manner.

To disrupt the *mxi1* open reading frame (ORF), we designed a positive/negative replacement-type vector that eliminates an exon required for the production of the two mouse Mxi1 isoforms[4] (Fig. 1a). On transmission of the *mxi1*-null allele through the germ line, heterozygous intercrosses yielded all three genotypes (Fig. 1b) at a ratio close to the expected Mendelian distribution (relative ratios $mxi1^{+/+}$ 1, $mxi1^{+/-}$ 2.5, $mxi1^{-/-}$ 0.9; $n = 118$). We used reverse transcription with polymerase chain reaction (RT-PCR), with a 5′-oligomer targeted to the deleted region, to confirm that the

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L5 473 S (DENDRITIC OR LANGERHAN#)(S)L2
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L72           3 S L71 AND PY<2000
L73           1 DUP REM L72 (2 DUPLICATES REMOVED)
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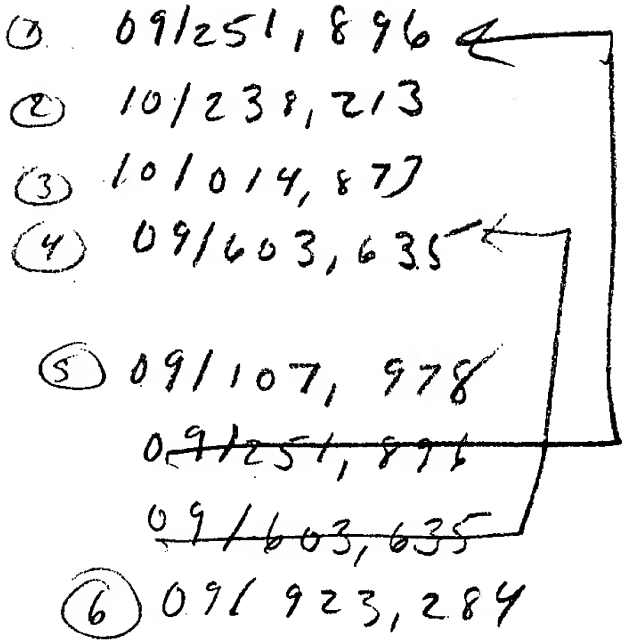

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PERIPHERAL DELETION OF MATURE CD8+ ANTIGEN-SPECIFIC T CELLS AFTER IN VIVO EXPOSURE TO MALE ANTIGEN[1]

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It has been well established that T cell tolerance to self Ag occurs primarily via clonal deletion of immature thymocytes in the thymus. Evidence also exists that there are additional mechanisms operative on mature T cells for establishing and maintaining tolerance in the periphery. To follow the fate of mature Ag-specific T cells in vivo, we used female transgenic mice, which contain a large population of male H-Y Ag-specific T cells that can be identified by immunostaining with mAb directed against CD8 and the transgenic TCR. H-Y Ag was introduced into these mice by injecting Ag-bearing male lymphocytes using conditions known to induce CTL precursor response reduction. The number of Ag-reactive CD8+ transgenic T cells in the periphery started to decrease after 2 days of in vivo exposure to male Ag. Decline was maximum (up to 80% of total) by 7 days, and stayed at this level for at least 6 wk. CD4+ cells and those CD8+ cells that did not carry the transgenic TCR were not affected. Most or all of the remaining Ag-reactive CD8+ cells in the periphery were fully responsive when stimulated by male Ag in vitro. Maturation of transgenic T cells in the thymus of injected mice remained the same as that of control animals. Our data provide direct evidence that mature Ag-reactive CD8+ cells are susceptible to clonal deletion in the periphery when exposed to the Ag in vivo. These findings suggest the presence of two types of APC in the periphery: stimulatory APC (e.g., macrophages and dendritic cells) required for initiating an active immune response; and functionally deleting APC (or veto cells) capable of deleting mature T lymphocytes that recognize Ag presented on their surface. Functionally deleting APC that present self Ag to peripheral T cells may provide a fail-safe mechanism against autoreactive cells that escaped deletion during differentiation in the thymus.

Intravenous administration of viable histoincompatible T lymphocytes can lead to a rapid reduction in the subsequent ability of the recipient to generate a CTL response against donor cells, while the response against third-party cells is not diminished (1-4). This specific reduction in response results from a rapid functional deletion of up to 90% of the reactive CTLp[3] (2-4). One explanation for this phenomenon is that the donor T lymphocytes act as functionally deleting APC (i.e., veto cells). Host CTLp specific for donor cell Ag would then receive negative deletional signals by interacting with donor cells.

Previously, it has been difficult to determine the fate of CTLp that have undergone functional deletion in vivo, i.e., whether they have been rendered anergic (5, 6), actually physically deleted (7, 8), or sequestered in some site outside the recirculating pool (9). These possibilities are difficult to distinguish from each other because the functionally deleted CTLp represent only a small fraction of the total T cell population, and there is no marker with which this population can be identified a priori. In this report, these two problems have been overcome by using cells from a previously described transgenic mouse carrying TCR-α and TCR-β transgenes isolated from a CD8+ CTL clone specific for the minor H-Y male Ag restricted by H-2D^b (10, 11). Transgenic female mice homozygous or heterozygous for the H-2^b haplotype develop a T cell repertoire that is skewed such that a large fraction of peripheral T cells expresses both CD8 and the transgenic TCR. The transgenic α- and β-chains can be detected with mAb T3.70 (12) and F23.1 (13), respectively.

We show here that such identifiable, Ag-specific mature T cells are deleted in the periphery, rather than being activated or rendered anergic, subsequent to encountering the H-Y Ag on male lymphocytes injected into host mice intravenously. These results are discussed in terms of mechanisms involved in inducing and maintaining peripheral tolerance to self Ag in mature T cells.

MATERIALS AND METHODS

Mice. C57BL/6 (B6) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). The genes for the α- and β-chains of a TCR that recognizes H-Y (male Ag) when presented by H-2D^b class I MHC molecule were introduced into mice (11), which were then backcrossed for 11 generations onto C57L mice (H-2^b). In these mice, the transgenic β-chain is expressed on all T cells but not all cells express the α transgene (10). Because the specificity for H-Y requires not only the transgenic α- and β-chains but CD8 molecules as well, male Ag-specific T cells are CD8+ with high level of expression of the α-chain TCR, which can be detected with anti-CD8 antibody and mAb T3.70 (12).

Reagents. mAb used were biotinylated or FITC-conjugated anti-

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[1] This work was supported by the Medical Research Council of Canada Grants MT-1540 (L.Z.), MT-3017 (R.G.M.), and MT-7123 (H.S.T.).
[2] Address correspondence and reprint requests to Dr. Richard G. Miller, Ontario Cancer Institute, 500 Sherbourne Street, Toronto, Ontario, Canada, M4X 1K9.

[3] Abbreviations used in this paper: CTLp, CTL precursors; LN, lymph node; LNC, lymph node cells.

CD8 (Becton-Dickinson, Mountain View, CA), phycoerythrin-conjugated anti-CD4 (Becton-Dickinson), biotinylated F23.1 (13), and biotinylated T3.70 (12). In the latter three, FITC- or phycoerythrin-conjugated streptavidin (Becton-Dickinson) was used as the fluorescence label.

Methods. For cytofluorometric analysis of blood samples, 20 μl of blood were collected with heparinized capillary tubes from the tail vein of each mouse before and at varying times after injection of male or female LNC. Blood samples were washed twice with $Ca^{-}Mg^{-}$ PBS and then incubated with mAb for 20 min at 4°C. These incubations were performed in the presence of 3% (v/v) mouse serum added to block nonspecific binding via FcR. After incubation with secondary antibodies, cells were washed, incubated with lysing solution (Becton-Dickinson) for 2 min, washed, and resuspended in 300 μl PBS containing 1% NaN_3 for FACS analysis. Single cell suspensions were also prepared from thymus, LN, or spleen. Two- or three-color surface staining of lymphocytes was performed as described (11, 12). The data were collected and analyzed using Consort 30 and FACScan software (Becton-Dickinson) on 50,000 events. Dead cells were excluded from data collection by setting a viability gate with forward and side-scatter parameters.

Cell suspensions used for i.v. injection were prepared from pooled axillary, inguinal, and mesenteric LN. When required, LNC were labeled with FITC as previously described (3). Cells labeled this way remain viable and can enter the recirculating pool after intravenous injection (3).

MLR. Various numbers of LNC (from 100 to 3×10^5/well) were seeded into U-bottomed 96-well plates as responder cells. Each responder concentration was set up in five replicates and incubated with 3×10^5/well irradiated (2000 rad) male or female C57BL/6 splenic cells. After 3 days of culture in α-MEM supplemented with antibiotics, 10% FCS, and 15% Con A supernatant (harvested from 3-day cultures of rat splenocytes with 2 μg/ml Con A), each well received 1 μCi of [^{3}H]TdR. Fifteen hours later, cultures were harvested and counted in a beta counter.

RESULTS

Female mice with T cells carrying the anti-male transgenic TCR were injected i.v. with B6 male LNC or, as controls, B6 female LNC. Before injection, and 2, 4, 7, 14, 21, 35, and 42 days after injection, blood samples were taken and lymphocytes were analyzed for expression of transgenic TCR, CD4, and CD8 by two-color flow cytometry. It is known that cells from the transgenic animal positive for CD8 or F23.1 (β-chain of TCR) need not necessarily be expressing the transgenic receptor, as they may have lost the transgenic TCR-α chain and be expressing an endogenous α-chain (11). However, all cells positive for the mAb T3.70 (reactive with the transgenic TCR-α chain) are expected to be expressing the transgenic receptor in association with CD8 (11). Therefore, plots of anti-CD8 vs T3.70 staining of blood samples at different measurement time points after injection of male cells were first examined.

These were divided into six regions as shown in Figure 1, which is a set of representative results from a single mouse before and 2, 4, 7, 21, and 28 days after injection of male LNC. Cells in region 1 are $CD8^{-}T3.70^{-}$; region 2, $CD8^{+}T3.70^{-}$; region 3, $CD8^{-}T3.70^{lo+}$; region 4, $CD8^{+}T3.70^{lo+}$; region 5, $CD8^{-}T3.70^{hi+}$; and region 6, $CD8^{+}T3.70^{hi+}$. Cell populations in regions 1, 2, and 3 remained unchanged at different time points. Region 4 showed a slight decrease 4 days after injection. Region 5 usually remained stable in population size but increased in some instances in a manner variable from mouse to mouse. Only $CD8^{+}T3.70^{hi+}$ cells in region 6 showed a significant decrease in all male cell-injected mice compared with control mice injected with female cells. Note that this region has been shown (12) to contain all the cells capable of proliferating in vitro in response to stimulation with male cells. After in vivo injection of male

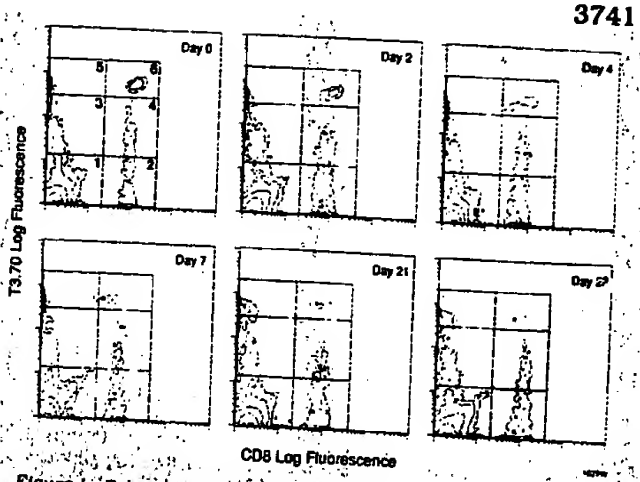

Figure 1. Deletion of Ag-specific $CD8^{+}$ T cells from PBL after in vivo exposure to male Ag. B6 male LNC were i.v. injected into a B6 H-Y transgenic female mouse. Before (day 0) and 2, 4, 7, 21, and 28 days after injection, PBL were collected and stained with anti-CD8 and anti-TCR-specific mAb (T3.70) as described in *Materials and Methods*. These two-color flow cytometry plots were divided into 6 regions numbered as in the top left: region 1, $CD8^{-}T3.70^{-}$; region 2, $CD8^{+}T3.70^{-}$; region 3, $CD8^{-}T3.70^{lo+}$; region 4, $CD8^{+}T3.70^{lo+}$; region 5, $CD8^{-}T3.70^{hi+}$; region 6, $CD8^{+}T3.70^{hi+}$.

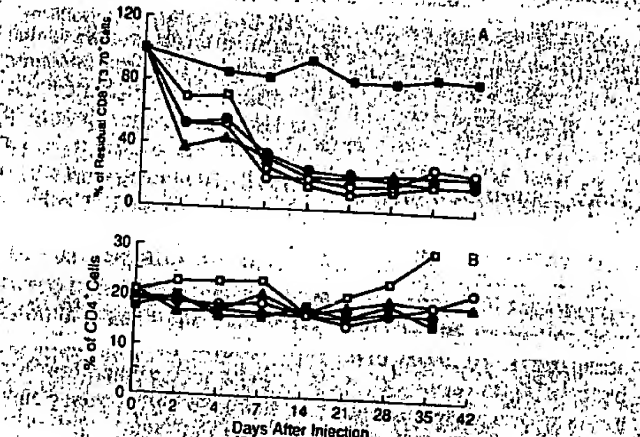

Figure 2. Dynamic changes of T cell subpopulations after injection of male Ag into H-Y female transgenic mice. B6 H-Y transgenic female mice were injected with LNC from B6 male (4 mice: □, ○, ▲, ●) or female (1 mouse: ■) mice. Before injection and 2, 4, 7, 14, 21, 28, 35, and 42 days after injection, blood samples were analyzed for the expression of CD8, T3.70, and CD4 by two-color flow cytometry as described in Figure 1. Data points correspond to the percentage of residual (original number = 100%) $CD8^{+}T3.70^{hi+}$ cells (*A*) and percentage of $CD4^{+}$ cells (*B*) in the PBL of each mouse at the time shown.

cells, cell number in this region fell sharply by day 2, was little changed on day 4, fell further by day 7 to about 20% of initial value, and stayed low for at least 6 wk (Fig. 2*A*). During this time there was little or no change in the number of $CD4^{+}$ cells (Fig. 2*B*) or indeed of any other population.

To examine whether the H-Y Ag-reactive T cells that have disappeared from PBL are sequestered in other lymphoid organs, LN, and spleen of male-injected and control mice were analyzed 6 wk after injection of male cells. It was found that the reduction of $CD8^{+}T3.70^{hi+}$ cells was even more pronounced in LN and spleen than in PBL (92.2% reduction in LN and 88.2% in spleen compared with controls). Results for one mouse of the group are shown in Figure 3. As was found in PBL, the $CD4^{+}$ population remained the same as in control (data not shown).

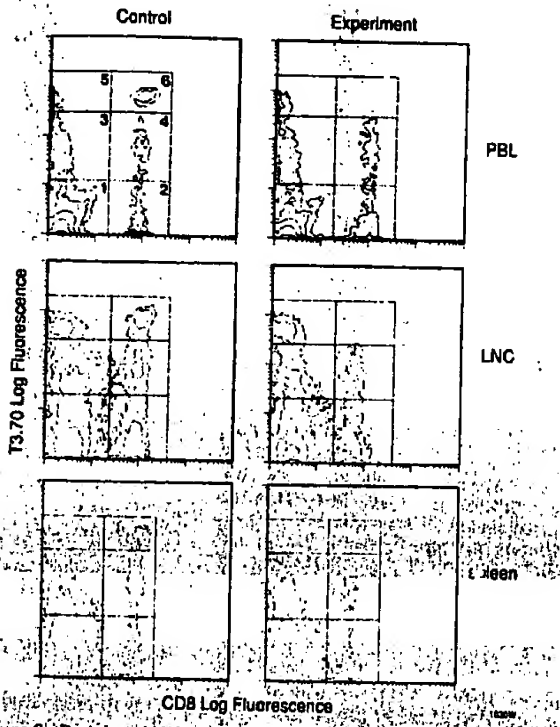

Figure 3. Peripheral deletion of male specific $CD8^{hi}$ cells in transgenic female mice. PBL, LNC, and spleen cells from H-Y transgenic female mice 6 wk after injection of female (*left*) or male (*right*) LNC were stained with anti-CD8 and anti-T3.70 mAb as described in Figure 1.

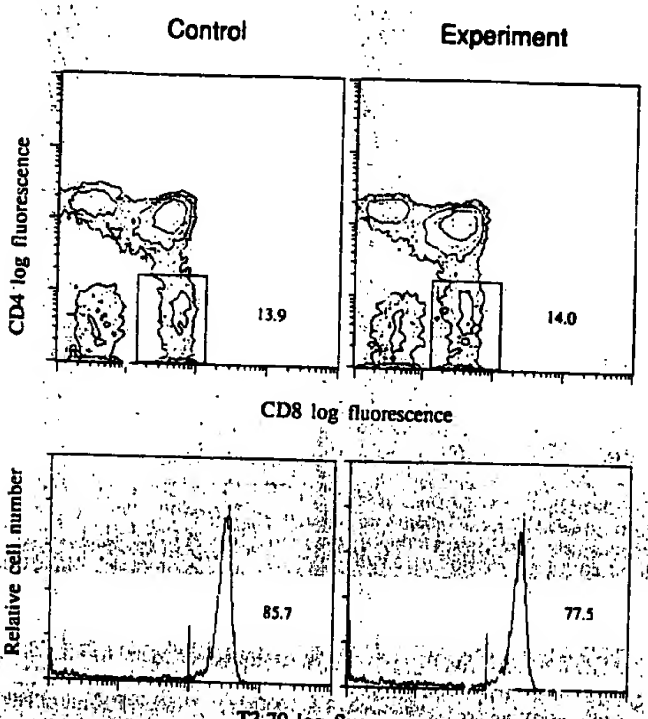

Figure 4. Lack of thymic deletion of male specific $CD8^+$ cells in mature female transgenic mice after in vivo exposure to male Ag. Thymocytes from H-Y female transgenic mice 6 wk after injection of female (Cont.) or male (exp.) LNC were analyzed by three-color flow cytometry for expression of CD4, CD8, and T3.70. At the *top* (CD4 vs CD8), the percentage of $CD4^-CD8^+$ cells is indicated. At the *bottom*, T3.70 staining of this subset is shown, with the percentage of $T3.70^+$ cells explicitly indicated.

Next, we tested whether injection of male cells affected thymic development of transgenic TCR-bearing cells. Thymocytes from female transgenic mice were analyzed by cell surface staining with antibodies specific for CD4, CD8, and transgenic TCR 6 wk after injection of male or female cells. Figure 4 shows that, in contrast to the periphery, there was no obvious difference between experimental and control mice in either the distribution of CD4 vs CD8 cells (*top*) or the relative number and intensity of $CD8^+T3.70^+$ cells (*bottom*). These data suggest that the reduction of mature H-Y Ag-reactive $CD8^+$ cells is induced and maintained in the periphery and that the thymus is unlikely to be involved in this peripheral deletion.

Because different individual female transgenic mice were observed to vary in their level of transgenic TCR expression, we adopted another design in which cells from a single transgenic donor could be used for both experimental and control mice. LNC from a single female transgenic mouse were labeled with FITC and injected i.v. into four or five nontransgenic recipient B6 female mice. After 1 day, a time sufficient to allow these cells to distribute homogeneously within the recirculating pool of the host (3), 1×10^7 B6 male LNC were injected i.v. into two mice in this group. The labeled injected transgenic cells would thus be exposed to the H-Y Ag that they recognize. The remaining two or three control mice were either not injected with additional cells or were given 1×10^7 nontransgenic female B6 LNC. All mice receiving cells from a given transgenic mouse were analyzed 2 or 4 days after injection of the male cells. Aliquots of LNC from each recipient were stained with phycoerythrin-conjugated (red fluorescence) mAb against CD4, CD8, F23.1, or T3.70 and analyzed by flow cytometry. By looking at the number of cells with each of these markers

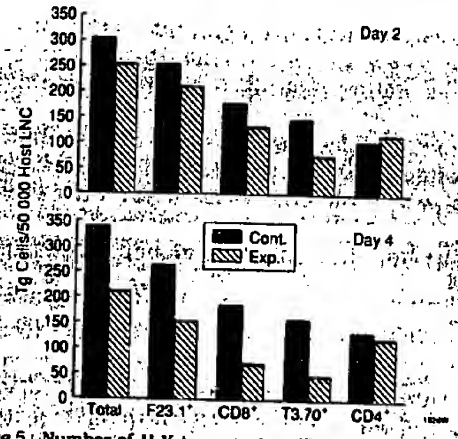

Figure 5. Number of H-Y transgenic cells in LNC recovered from B6 female mice injected with FITC-labeled female transgenic cells 2 and 4 days after injection of B6 male cells. Transgenic female LNC were labeled with FITC and then i.v. injected into nontransgenic female hosts. One day later mice also received either an injection of 1×10^7 female lymphocytes (control) or male lymphocytes (experiment), and 2 and 4 days later LNC were collected and labeled with either phycoerythrin-conjugated F23.1, CD8, T3.70, or CD4 mAb. Cells from the transgenic donor can be discriminated from host cells by the emission of green fluorescence. Each panel presents mean number of transgenic cells in an aliquot of 50,000 cells analyzed from two control (*solid bars*) and two experimental (*hatched bars*) mice. Similar results were obtained on analyzing spleen cells.

that were also FITC positive, the remaining number of injected cells carrying that marker could be determined.

As shown in Figure 5, there was a significant decrease, more obvious with time, in the total number of injected transgenic cells in mice injected with male cells compared with the control group (120 ± 19 per 5×10^5 on day 4). This drop is matched by a comparable drop in the number

of $CD8^+$, F23.1$^+$, and T3.70$^+$ cells (106, 104, and 105, respectively, all per 5×10^5 on day 4) consistent with all or most of the cells that had disappeared being $CD8^+$ cells carrying the transgenic receptor reactive against H-Y. When $CD4^+$ cells were analyzed in the same manner, no significant dynamic change could be observed (11 ± 14 on day 4). These results are fully concordant with those from the first design.

Because the elimination of $CD8^+$T3.70$^+$ cells in the present study was not complete, the question arises whether the 20% residual nondeleted male reactive cells were rendered anergic. After 6 wk of in vivo exposure to male or female lymphocytes, LNC from female transgenic mice were collected and stimulated by irradiated male splenocytes in an MLR. Figure 6 shows results for the remaining male-reactive $CD8^+$ cells from three mice injected with male cells. One (*top*) responded identically to control, suggesting that all remaining $CD8^+$T3.70$^+$ cells were fully responsive. The other two (*bottom*) were a little (approximately twofold) less responsive than control.

DISCUSSION

Although the concept that clonal deletion is a major mechanism of tolerance induction has been well established at the level of immature T cells in the thymus, the mechanisms involved in mature T cell tolerance remain controversial (6, 14–21).

In this report viable B6 male LNC were introduced into female transgenic mice expressing TCR specific for the male H-Y Ag presented by H-2 D^b class I MHC molecules. The fate of mature male H-Y Ag-reactive $CD8^+$ T cells was monitored with mAb specific for the transgenic TCR and by in vitro functional assay. It was found that in vivo exposure to male Ag led to disappearance of up to 80% of the mature $CD8^+$T3.70$^+$ Ag-reactive T cells for at least 6 wk. These data, together with the fact that most or all of the remaining male reactive cells in mice injected with male lymphocytes remained responsive, suggest that the majority of mature Ag-reactive T cells in the periphery have undergone deletion.

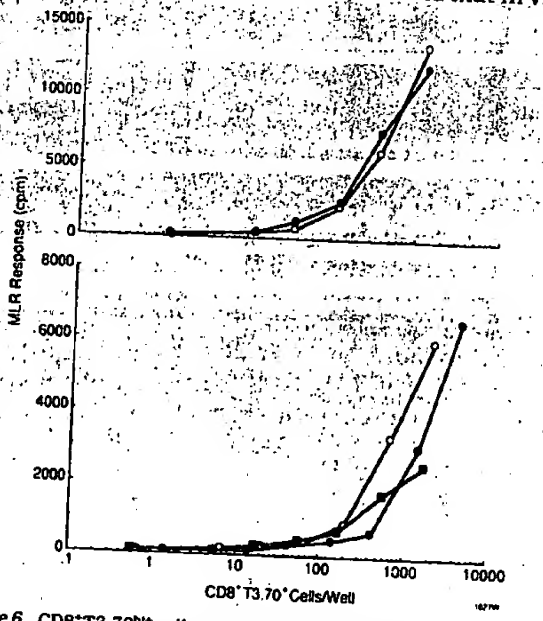

Figure 6. $CD8^+$T3.70^{hi+} cells remaining in male injected mice are fully responsive. LNC from female H-Y transgenic mice 6 wk after injection with male (*closed symbols*) or female (*open symbols*) cells were stimulated by irradiated male cells in an MLR and proliferation ($[^3H]$TdR incorporation, cpm) measured after 3 days. Results are plotted as cpm vs number of $CD8^+$T3.70$^+$ cells/culture. The two panels are from two independent experiments, the top testing one experimental and one control mouse, the bottom testing two experimental and one control mouse. Each data point represents a mean of five replicates. The responses in control cultures using female stimulator cells were at least 10-fold lower (data not shown).

All transgenic mice analyzed contained a population of $CD8^+$T3.70^{lo+} cells (Fig. 1, region 4), which was unaffected by exposure to male LNC despite the fact that it appears to have been selected as specific for male Ag. A possible explanation is as follows. It is known that mice carrying the anti-H-Y transgenic TCR do not rearrange endogeneous TCR-β chains but can, on occasion, rearrange endogeneous TCR-α chains (11, 12). Furthermore, some T cells can then express two different TCR, one using the transgenic TCR-α chain (identifiable with anti-T3.70 mAb) and the other an endogeneous TCR-α chain (22), both competing for available TCR-β chain. Cells of this type probably correspond to the population of $CD8^+$T3.70^{lo+} cells. We hypothesize that the level of anti-H-Y TCR expression is too low to trigger either activation or removal.

Earlier reports (23–26) have indicated that injection of Ag that have a propensity for accumulating in the spleen may lead to selective recruitment of Ag-reactive lymphocytes from the recirculating pool to the spleen. However, the observed accumulation of Ag-reactive lymphocytes appears to be a temporary phenomenon, persisting for only approximately 2 days (25, 26). It appears unlikely that a similar phenomenon could account for the selective disappearance of H-Y Ag-reactive transgenic cells for several reasons: 1) we have previously shown that i.v. injected lymphocytes homogeneously distribute in the host spleen and LN (3); 2) the distribution of Ag-reactive CTLp is not altered in the host lymphoid tissues subsequent to an i.v. injection of allogenic cells (3); and 3) We have shown here that H-Y Ag-reactive T cells disappeared not only from PBL but also from LN and spleen on exposure to their Ag on lymphoid cells. The above arguments suggest that it is unlikely that H-Y reactive cells are being sequestered within one particular compartment of the lymphoid tissues. However, we cannot definitively exclude the possibility that Ag-reactive cells are being sequestered in nonlymphoid tissues outside the recirculating pool.

Recently, it was reported that peripheral tolerance can be achieved by down-regulating TCR and CD8 expression (19, 20). Because our analysis has mainly focused on $CD8^+$T3.70^{hi+} cells, one might argue that male H-Y Ag-specific $CD8^+$ cells are still in the periphery but no longer detectable as $CD8^+$T3.70$^+$ bright cells. If the reduced level of male H-Y Ag-reactive cells was due to down-regulation of TCR and CD8 molecules on the cell surface, we should be able to see an increased proportion of low intensity and/or $CD8^-$T3.70$^-$ cells. Yet when we carefully checked on the FACScan, there was no increase, but instead a slight decrease, in the proportion of lymphocytes expressing low levels of the transgenic TCR (Figs. 1 and 3). In addition, the intensity of staining (Fig. 1) and the reactivity (Fig. 6) of the residual $CD8^+$T3.70$^+$ cells appeared normal or nearly so. These results suggest that it is unlikely that the reduction of male H-Y Ag-reactive T

cells is the reflection of down-regulation of TCR expression.

It is noteworthy that the removal of male H-Y Ag-reactive T cells was only partial (80%) and not complete. This level of reduction stayed constant over at least 6 wk, suggesting that newly produced transgenic receptor-bearing T cells continued to be deleted. In previous studies, we found that when F_1 (C57BL/6 × DBA/2) lymphoid cells were injected i.v. into C57BL/6 mice, again ~80% of CTLp reactive against DBA/2 were inactivated (3). When naive C57BL/6 lymphoid cells were introduced into the injected mice at later times (up to at least 2 wk), about 80% of CTLp in the naive cells were also inactivated, suggesting that the ability of the F_1 injected cells to produce response reduction persists for considerable time (27). We speculate that a subset of the T cells are resistant to the inactivation mechanism. It is of note that it has previously been shown that memory T cells are not sensitive to the action of veto cells in vitro (28–30).

Active suppression has been widely proposed as a mechanism for establishing and maintaining peripheral tolerance. According to this model, autoreactive T cells might continue to persist in the periphery, but their activation would be prevented by a specific immunoregulatory T cell population. Here, we report the peripheral disappearance of male H-Y Ag-reactive cells starting within 2 days of exposure to male cells, a time that would appear to be too short for the induction of specific Ts cells. Furthermore, in a study of the response reduction produced by i.v. injection of viable histoincompatible lymphocytes, we found that up to 6 days after injection of cells, donor cells retrieved from the recipient, but not recipient cells, could transfer response reduction to a new recipient (3).

Studies by Rammensee et al. (6) have shown that Mls-reactive lymphocytes are rendered anergic in vivo on encountering Mls-bearing lymphocytes, which they can recognize. The cells persist but can no longer be activated. Anergy of Mls^a-reactive mature T cells has also been observed in chimeras (14, 15) and in TCR transgenic mice (31), strengthening the view that clonal deletion operates only at the level of immature T cells. However, in our studies, the physical disappearance of Ag-specific T cells from the periphery is clearly not clonal anergy. Furthermore, the remaining male-specific T cells in the periphery are largely responsive to male Ag, also arguing against clonal anergy. Webb et al. (16) have reported that Mls^b mice injected with Mls^a $CD8^+$ cells underwent a 60 to 80% reduction in the number of Mls^a-reactive $V\beta6^+$ cells in spleen and LN relative to uninjected control mice, with little or no decrease in the proportion of Mls^a-nonreactive $V\beta8^+$ cells. Their findings are concordant with our results in the sense of peripheral deletion of Ag-specific T cells. However, instead of $CD8^+$ cells, they found that deletion was only apparent at the level of $CD4^+$ cells. Note that Mls-reactive cells are $CD4^+$ and recognize Ag in association with class II MHC, whereas our study concerns $CD8^+$ cells that recognize Ag in association with class I MHC. Because these two T cell families are biologically very different, one being primarily concerned with immunoregulation through lymphokine production and the other being primarily cytotoxic effector cells, it is perhaps not unreasonable that peripheral tolerance might be established by different mechanisms.

Recently, Rocha and von Boehmer (19) injected male-specific H-Y transgenic T cells into B6 nu/nu male mice. They found that male-specific cells first underwent pronounced clonal expansion. After day 5, cell number fell precipitously, suggesting deletion. The remaining cells were anergic on in vitro assay, perhaps as a result of having down-regulated both TCR and CD8 expression. These results differ markedly from ours. We suggest this may be due to differences in the way in which male-specific transgenic T cells encounter H-Y Ag. In the Rocha and von Boehmer system (transgenic T cells from female mouse transferred to male nude mouse), the transgenic T cells can easily encounter highly stimulatory H-Y bearing APC such as dendritic cells or macrophages but are unlikely to encounter H-Y bearing lymphocytes that might act as veto cells. In our system (normal male lymphoid cells transferred into female mice carrying anti-H-Y transgenic T cells) exactly the reverse is true, i.e., transgenic T cells are unlikely to encounter stimulatory APC bearing H-Y Ag but will easily encounter H-Y bearing lymphoid cells. Thus, the two systems are complementary. The differences in results suggest there are several different mechanisms for achieving peripheral tolerance.

Both Webb et al. (16) and Rocha and von Boehmer (19) observed marked clonal expansion before specific deletion of Ag-specific cells. This was not observed in our system, perhaps because of the differences noted above. However, we did observe that on day 2 and, especially, on day 4 after infusion, there were blast cells in PBL, as indicated by a cell subpopulation with increased forward and side scatter. When different lymphocyte subsets were gated to see what kind of cells were activated, it was found that neither $CD8^+T3.70^+$ nor $CD4^+$ cells were becoming blast cells. Instead, $CD4^-CD8^-T3.70^-$ (B cells or $CD4^-CD8^-$ T cells) in PBL showed a clear transient activation (data not shown). This began on day 2, reached maximum on day 4, and disappeared by day 7. At present, the biologic significance of these cells is unknown.

Because the transgenic, Ag-specific $CD8^+$ T cells are known to recognize H-Y Ag (10), it must be concluded that the manner in which they initially encounter Ag can determine their subsequent fate. An encounter with male H-Y Ag presented on the surface of, for example, spleen-derived dendritic cells or macrophages, can result in stimulation of the Ag-reactive cells (10). On the other hand, as shown here, encounter with Ag on recirculating male LNC results in clonal deletion. Taken together with earlier findings (2–4, 28–30) these observations are consistent with the existence of functionally deleting APC (veto cells).

It has been postulated that the same antigenic structure must be capable of producing either T cell activation or T cell tolerance, the result obtained depending upon the differentiation state of the T cell and/or the nature of the cell presenting the Ag (cf. Refs. 5 and 8). Recent evidence strongly supports the view that immature T cells, at the stage in development when they express both CD4 and CD8, die (undergo clonal abortion) upon recognizing APC (7, 8, 10). These same APC can activate more mature T cells bearing only CD4 or CD8 (8). Thus, developing T cells pass through a state in which a signal that would be stimulatory for a mature T cell instead induces tolerance. This process appears to occur primarily in the

thymus. Here, in contrast, we are observing peripheral deletion of Ag-specific, mature CD8 cells as a result of the nature of the APC.

Although the data are consistent with the existence of a deletional APC in the periphery, it remains possible that the deletion of $4^-8^+T3.70^+$ cells is due to the lack of a costimulatory or helper signal after engagement with male APC. This idea is more in line with the Bretscher/Cohn 2 signal model where the Ag-reactive cell dies if it does not receive the second signal (32). The lack of a second signal in this situation could be due to inefficient Ag presentation to Th cells by the recirculating male cells and/or the low frequency of helper cells that can recognize the male Ag.

In the male environment where H-Y Ag is a self Ag, clonal deletion could be important for maintaining vigil against autoreactive clones that escape clonal abortion during development in the thymus or against T cells that may have differentiated through a thymus-independent pathway, as evidenced in the nude mouse (33). The balance, and possible antagonism, between stimulatory and deletional Ag presenting mechanisms becomes an interesting and challenging problem for study, particularly with the implication that such contests may be at the root of certain disease mechanisms and involved in processes of specific tolerance induction.

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Inhibition of Langerhans Cell Antigen-Presenting Function by IL-10

A Role for IL-10 in Induction of Tolerance[1]

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Abstract. IL-10 is a product of activated keratinocytes and is released during the induction phase of contact sensitivity. As IL-10 effects have been described as being mediated by APC, we investigated effects of IL-10 on epidermal Langerhans cells (LC), the resident APC in the epidermis. Initial studies failed to demonstrate effects of IL-10 on MHC class II Ag expression by LC or anti-CD3 mAb- or alloantigen-induced LC-dependent T cell proliferation. However, production of IFN-γ and IL-2, (but not IL-6) was markedly reduced in these assays. When the soluble-protein Ag-specific T cell clones AE7 (Th1) and D10.G4 (Th2) were substituted for unprimed T cells, differential effects of IL-10 on T-cell proliferation were observed. Whereas IL-10–pretreated and untreated LC supported Th2 cell proliferation equally well, IL-10–pretreated LC were essentially unable to induce Th1 cell proliferation in response to native protein or peptide Ag. The inhibitory influence of IL-10 on Th1 cells was observed when fresh or 1 day cultured LC were used; 2- or 3-day cultured LC were affected to a much lesser extent by IL-10 pretreatment. Further, coculture experiments using IL-10–pretreated or untreated LC of a different haplotype suggest that IL-10 negatively regulates a costimulatory signal required for induction of Th1 cell proliferation. To assess whether T cells incubated with Ag and IL-10–pretreated LC were responsive to further stimulation, T cells were rescued after 1 day of coculture with IL-10–pretreated LC and restimulated, either immediately or after 1 to 5 days of rest, with untreated LC in the presence of Ag. T cells incubated with IL-10–pretreated LC were found to be anergic, whereas T cells incubated with untreated LC proliferated normally after further stimulation. However, anergic T cells responded vigorously to IL-2. These data indicate that although IL-10–pretreated LC are effective APC for Th2 cells, they fail to induce Th1 cell proliferation and rather induce clonal anergy in these cells. *Journal of Immunology*, 1993, 151: 2390.

IL-10 was originally identified as a Th2 cell product that inhibited the proliferation of Th1 cell clones by downregulating IFN-γ and IL-2 production (1, 2). This effect was shown to be dependent on the presence of viable APC and was observed only when macrophages were used as APC (3). Subsequently, other cell types including B cells (4), mast cells (5), and monocytes (6) were shown to be sources of IL-10, and a spectrum of additional IL-10 activities was identified. It is now known that IL-10 can act as a growth cofactor for mature and immature T cells (7), as a differentiation factor for cytotoxic T cells (8), as a growth factor for B cells [especially Ly-1$^+$ B cells (9)], and as a cytokine synthesis inhibitory factor in activated monocytes (6). In addition, IL-10 can enhance MHC class II expression on B cells (4), whereas it inhibits MHC class II expression on monocytes (10). Interestingly, reversal of the IL-10–induced inhibition of MHC class II expression on monocytes by IL-4 did not reverse the effect of IL-10 on T cell proliferation, suggesting an effect of IL-10 on monocytes distinct from inhibition of MHC class II molecules.

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Keratinocytes, which comprise the vast majority of epidermal cells, also produce IL-10 (11). IL-10 production by keratinocytes is markedly enhanced after application of contact sensitizers like trinitrochlorobenzene, whereas irritants or tolerogens do not enhance IL-10 mRNA or protein production. IL-10 mRNA signal strength in response to Ag was found to increase at 4 to 6 h, reaching maximal strength 12 h after allergen application compared with other cytokine mRNAs such as IL-1β that are increased within 15 min. The increase in IL-10 mRNA signal strength was accompanied by abrogation of T cell derived IFN-γ and interferon-induced protein-10 (IP-10) mRNA signals (12), suggesting a possible counterregulatory role for IL-10 in delayed-type hypersensitivity reactions in skin. Because effects of IL-10 on cytokine production by T cells have been shown to be mediated by metabolically active APC (1–3), we determined whether the Ag-presenting functions of epidermal LC[4] could be influenced by IL-10. Here we show that IL-10 inhibits the APC function of LC for Th1 but not TH2 cells. This inhibition occurred to a much lesser extent if LC were cultured for 2 to 4 days before IL-10 treatment. Additionally, we demonstrate that IL-10–pretreated LC induce lasting clonal anergy in Th1 cells rescued from original cultures and restimulated in the absence of IL-10. This effect was not mediated via downregulation of MHC class II expression on LC, suggesting an effect of IL-10 on other costimulatory factors.

Materials and Methods

Animals

C3H/HeN, C57/BL6, and BALB/c mice were obtained from the Charles River Breeding Laboratory, Wilmington, MA.

Cell lines

The pigeon cytochrome c specific, I-E^k-restricted Th1 clone AE7 (13, 14) was kindly provided by Dr. B. Beverly (NIAID, Bethesda, MD), the purified protein derivative (PPD)-specific H-2^d restricted Th1 clone LNC.2 was kindly provided by Dr. E. Schmitt (Intitut für Immunologie, University of Mainz), and the conalbumin specific I-A^k-restricted Th2 clone D10.G4 (15) was kindly provided by Dr. R. Hodes (NCI, Bethesda, MD). The clones were grown in RPMI 1640 containing 10% FBS (Biofluids, Rockville, MD), 2 mM glutamine (GIBCO Laboratories, Chagrin Falls, OH), 1% penicillin/streptomycin/fungizone solution (GIBCO), 1% nonessential amino acids solution (GIBCO), 1% sodium pyruvate solution (GIBCO), 10mM HEPES buffer solution (GIBCO), 5×10^{-5} M 2-ME, and 1 μg/ml indomethacin (Sigma Chemical Co., St. Louis, MO). This medium is referred to as complete medium. Cells were maintained in a rest/stimulation protocol as described (14). During rest periods, media was supplemented with 15 U/ml IL-2 (Genzyme, Boston, MA).

Serologic reagents

The following mAb directed against murine Ag were used: 10.2.16, specific for I-A^k from ATCC, Rockville, MD; M5/114.5.2 specific for I-A^{b,d,q} and I-E^{d,k} (ATCC); monoclonal 18.5 anti-rat κ (ATTC); 53.6.72 (anti-CD8, ATCC); as well as appropriate subclass controls from Becton-Dickinson, Mountain View, CA. Goat anti-rat or goat anti-mouse FITC F(ab)$_2$ (TAGO, Burlingame, CA) were used as second-step reagents.

Preparation of cell suspensions and EC culture

EC suspensions and lymph node T cells were prepared as previously described (16). EC suspensions were passed over Lympholyte M density gradients (Cedarlane, Ontario, Canada) and centrifuged at 300 $\times$ g for 10 min at room temperature either immediately after trypsinization or after 1 to 4 days of culture in complete medium. Interface cells were washed extensively in complete medium and either stained for FACS analysis or used in functional assays. These cells were enriched to contain 20 to 25% LC and are referred to as LC. Although the remaining cells in the epidermal cell preparation are keratinocytes (mainly), melanocytes, Merkle cells and dendritic Thy1$^+$ epidermal cells, none of these populations function as APC. LC were treated with rmIL-10 (100 U/ml) or equivalent amounts of mock transfectant COS cell supernatant (both kindly provided by Dr. K. Moore, DNAX, Palo Alto, CA) as indicated. In some experiments, a mAb specific for IL-10 (or a control mAb) was added to the cultures at a concentration of 10 μg/ml (PharMingen, San Diego, CA).

Allogeneic proliferation assay

T cells from BALB/c mice were prepared from peripheral lymph nodes (including mesenteric lymph nodes) by nylon wool columns as described (17) and depleted of APC and CD8$^+$ T cells by treatment with M5/114 and 53.6.72 mAb followed by anti-rat κ (18.5) and complement (Cedarlane). T cells (>95% CD4$^+$) were cultured for 96 h with varying numbers of LC derived from C3H or C57/BL 6 mice in the presence or absence of IL-10 or control supernatant. During the final 12 to 16 h of culture, [^{3}H] TdR (1 μCi/well) was added to the cultures. Cells were subsequently harvested using a semiautomated cell harvester (Cambridge Inc., Cambridge, MA), and incorporation of [^{3}H]-TdR was measured by liquid scintillation counting. Data are expressed as the arithmetic mean cpm $\pm$ SEM.

[4] Abbreviations used in this paper: LC, Langerhans cells; PCR, polymerase chain reaction; EC, epidermal cells; cpm, counts per min.

Anti-CD3 proliferation assay

Lymph node $CD4^+$ T cells were prepared as described above and mixed with varying numbers of LC that were either pretreated with 100 U/ml rmIL-10, control (mock COS supernatant), or left untreated. T cell receptor cross-linking was achieved by addition of 1% 145-2C11 (anti-CD3, from J. Bluestone, University of Chicago) culture supernatant (18). [^{3}H]-TdR (1 μCi/well) was added for the last 6 h of the 48 h culture period, and cells were harvested and counted as described.

Proliferation of H-2^k T cell clones

After resting for more than 14 days in complete medium supplemented with 15 U/ml rIL-2, the responder clones AE7 or D10.G4 were cocultured for 64 to 70 h with varying numbers of IL-10- or control-pretreated or untreated fresh or cultured LC with or without Ag. Responder cells were depleted of MHC class II-bearing cells and $CD8^+$ T cells as described above before initiation of the culture. [^{3}H]-TdR was added for the final 12 to 16 h of culture. In some experiments, anti-IL-10 mAb (PharMingen) or control mAb was added at 10 μg/ml to the cultures.

Costimulation assay

BALB/c EC suspensions were prepared as described and cultured for 3 days in the presence or absence of 100 U/ml IL-10. Cells were enriched for LC on day 3 by density gradient centrifugation (≈20%) and cocultured (5×10^4/well) with freshly-prepared 10^5 C3H LC (≈10% LC) pulsed with cytochrome c in the presence of 100 U/ml IL-10 and 2×10^4 AE7 responder cells. Cells were harvested after 60 h of culture; [^{3}H]-TdR (1 μCi/well) was added during the last 16 h of culture. Controls for this experiment are described in *Results*.

Induction of clonal anergy

In an attempt to induce clonal anergy, LC were precultured overnight with either medium alone, control supernatant, or 100 U/ml IL-10 in the presence of Ag. Nonadherent cells were subsequently harvested and enriched for LC by passage over Lympholyte M gradients as described. Enriched preparations routinely contained 20 to 25% LC as determined by FACS. LC fractions then were cocultured with AE7 responder cells (2×10^5/well) at a density of 5×10^5 cells/well in 24-well plates (Costar, Cambridge, MA) for 16 to 20 h (19). After coculture, T cells were rescued by passage over density gradients (Histopaque 1.077, Sigma) and either restimulated immediately or after rest periods of 1 to 5 days during which they were cultured in complete medium containing 2 U/ml IL-2. Rescued T cells (4×10^4) were restimulated with 2.5×10^4 fresh LC in the presence of Ag in 96-well plates. After 48 h of culture, 1 μCi/well [^{3}H]-TdR was added for the last 16 h. To determine the proliferation capacity of all rescued T cells, 100 U/ml IL-2 were added to 4×10^4 T cells and ^{3}H-TdR incorporation was assessed as above. Further controls are defined in *Results*.

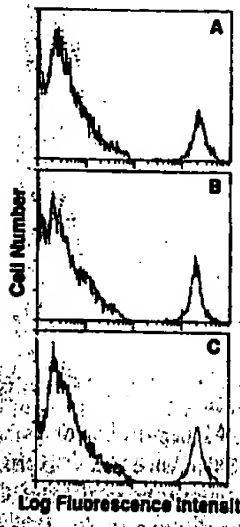

FIGURE 1. IL-10 pretreatment of LC does not affect their MHC class II expression. Epidermal cell suspensions were prepared and cultured overnight with (*A*) medium, (*B*) mock-COS supernatant, or (*C*) 100 U/ml IL-10. After culture, LC were enriched by density gradient centrifugation, stained with antibodies to MHC class II Ag (*solid lines*) or with control antibodies (*stippled lines*) and analyzed by FACScan.

PCR and liquid hybridization

RNA from cocultures of T cells and LC was extracted from anti-CD3 assays using IL-10 treated or untreated LC by the guanidinium thiocyanate/CsCl method as described for IL-2 and IFN-γ (20) or as modified for B7 mRNA[5]. RNA was quantitated spectrophotometrically, reverse transcribed (Superscript RT, BRL, Gaithersburg, MD), and subjected to 25 cycles of PCR as described earlier (12). PCR products were then hybridized to internal, ^{32}P-end-labeled oligonucleotides in fluid phase, electrophoresed on 4% PAGE, and detected by autoradiography as described (12). As labeled probes were added in excess, more specific PCR product results in increased signal strength on the gel but only in the linear amplification range of the PCR. Therefore, standard curves for primer concentration, amounts of RNA, and cycle numbers were established to ensure linearity as described (12). Signal strength of each individual cytokine can be compared only with its own baseline. Primer sequences were as described previously (12).

[5] M.G. Lee, T. Borkowski, and M.C. Udey. Regulation of expression of B7 by murine Langerhans cells: a direct relationship between the level of surface expression of B7 by Langerhans cells and B7 mRNA levels. *Submitted for publication.*

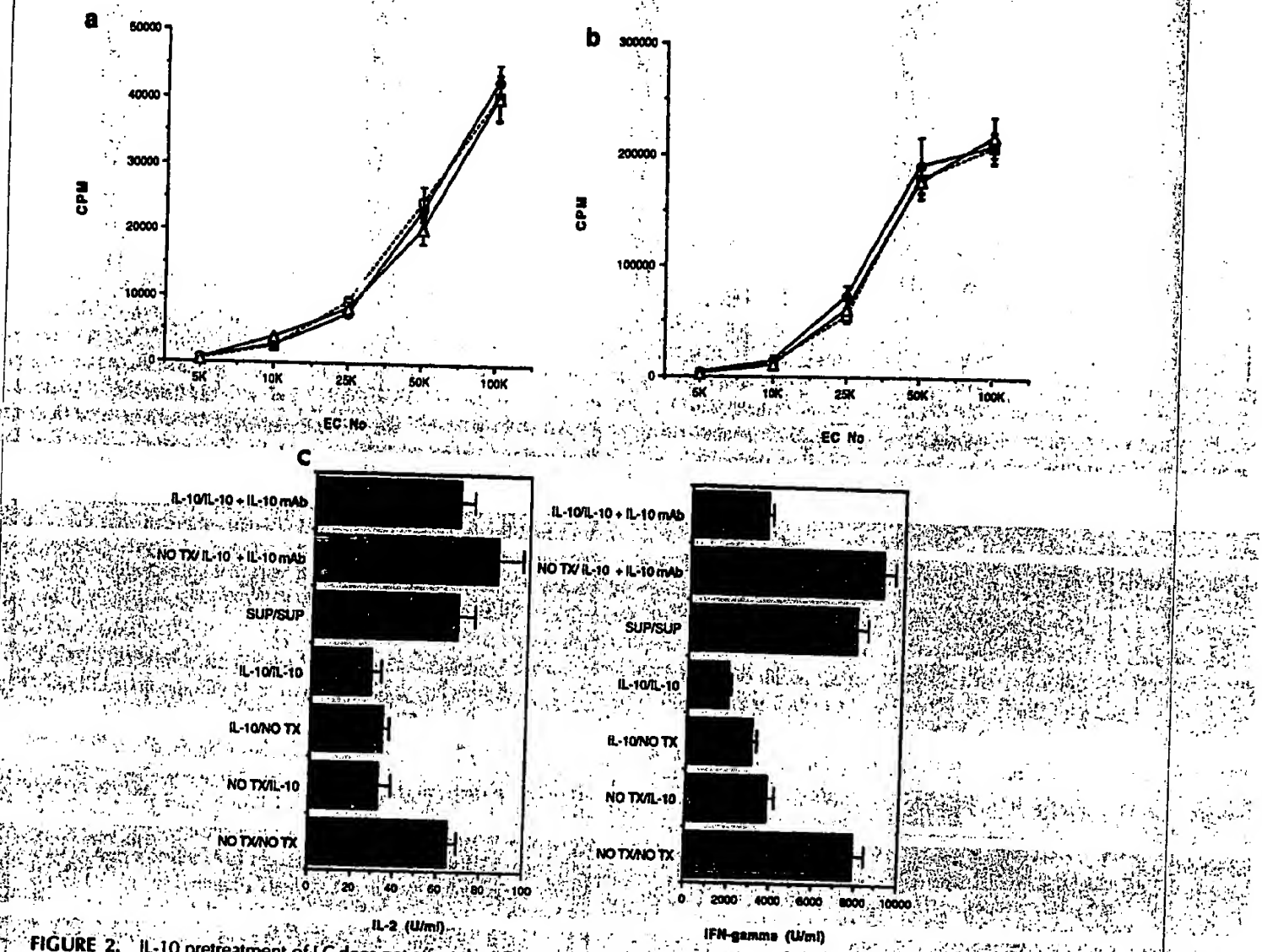

FIGURE 2. IL-10 pretreatment of LC does not affect the LC-supported allogeneic or anti CD3-response of lymph node CD4+ T cells. EC suspensions from C3H or C57/BL6 were cultured for 1 day either in medium (*open squares*), mock supernatant (*open triangles*), or 100 U/ml IL-10 (*closed circles*) and enriched for LC (15 to 20%). *a*, Cells were cocultured with BALB/c T cells for 96 h and 1 μCi/well ^{3}[H]-TdR was added for the last 18 h. *b*, BALB/c EC suspensions were pretreated overnight with either medium (*closed circles*), 100 U/ml IL-10 (*open triangles*), or mock supernatant (*open squares*). They then were cultured with BALB/c T cells and anti-CD3 mAb. ^{3}[H]-TdR was added at 1 μCi/well for the last 4 to 6 h of the 48 h culture period. Supernatants from anti-CD3 proliferation assays were harvested after 2 days and analyzed by ELISA for IFN-γ or IL-2. *c*, Groups were supernatants from untreated cells (NO TX/NO TX), untreated EC cocultured with T cells in the presence of 100 U/ml IL-10 (NO TX/IL-10), IL-10–pretreated EC cocultured without IL-10 (IL-10/NO TX), EC cultured in the continuing presence of IL-10 (IL-10/IL-10), EC pre- and cocultured with mock supernatant (SUP/SUP), untreated EC cocultured with T cells in the presence of IL-10 and 10 μg/ml IL-10 mAb (NO TX/IL-10 + IL-10 mAb), and IL-10–pretreated EC cocultured with T cells in the presence of IL-10 and IL-10 mAb (IL-10/IL-10 + IL-10 mAb).

Reagents

Commercially available ELISA kits were used for determination of IL-2 (Collaborative Biomedical Products, Bedford, MA), IL-6 (Endogen, Boston, MA), and IFN-γ (Genzyme) following the instructions of each manufacturer.

Results

Effects of IL-10 on MHC class II expression of epidermal LC

As IL-10 exerts a profound influence on the expression of MHC class II cell surface molecules on certain cell types,

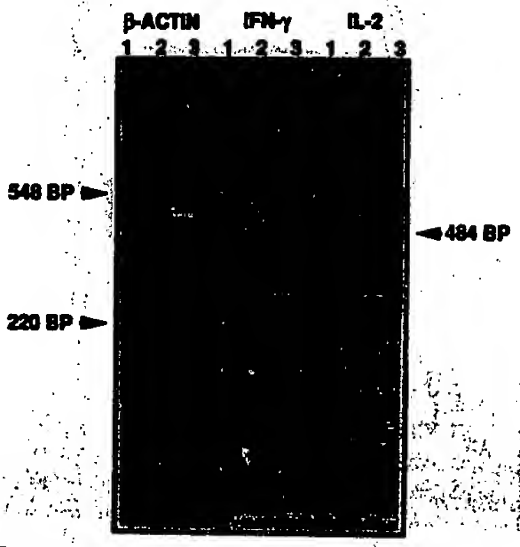

FIGURE 3. Effect of IL-10 on IFN-γ, IL-2, and β-actin mRNA. EC and peripheral lymph node T cells were cocultured for 48 h in 24-well plates in the presence of anti-CD3. At the end of the culture period, RNA was extracted with guanidinium thiocyanate and subjected to 25 cycles reverse transcriptase-PCR followed by liquid hybridization. Products then were run on 4% PAGE, dried, and autoradiographed. Cells were cultured in the presence of 1, no treatment; 2, mock COS supernatant; or 3, 100 U/ml IL-10.

we determined whether similar changes were induced on LC. EC suspensions were prepared as described and cultured in the presence or absence of IL-10 (100 U/ml) or control supernatant for 1 to 3 days. Nonadherent cells were enriched for LC by density-gradient centrifugation and stained with 10.2.16 mAb (anti-I-A^k) (or control) and FITC $F(ab)_2$ goat anti-mouse IgG. IL-10 had no effect on LC MHC class II expression or viability of cells treated for 1, 2, or 3 days (Fig. 1).

Effect of IL-10 on LC-dependent anti-CD3 or alloantigen-induced $CD4^+$ T cell proliferation

To assess whether IL-10 affects LC in a fashion similar to macrophages and monocytes, LC were pretreated either with IL-10 (100 U/ml), mock supernatant, or medium alone and used as APC in anti-CD3 or alloantigen-induced proliferation assays using lymph node $CD4^+$ T cells as responders. IL-10 pretreatment of LC did not affect the T cell proliferative response (Fig. 2, *a* and *b*). When supernatants from these anti-CD3–containing cultures were tested for IL-2 or IFN-γ (at the highest ratio of LC and T cells) by ELISA, both were found to be significantly reduced in IL-10–treated cultures compared with control. The reduction was most striking when LC were pretreated with IL-10 for 24 h and was observed even if IL-10 was subsequently omitted from the proliferation assays. Even without LC-pretreatment, there was a significant reduction in IL-2 and IFN-γ production when IL-10 was added to the coculture of LC and T cells (Fig. 2*c*). Addition of IL-10 mAB completely reversed the inhibitory effect of IL-10 on IL-2 production. IL-6 production was not affected thus excluding a nonspecific effect of IL-10 on Th2-derived cytokines.

Effect of IL-10 on cytokine mRNA signals

To determine whether the effect of IL-10 pretreatment of LC on cytokine synthesis by T cells occurred at the transcriptional level, total RNA of LC/T cell cocultures was extracted on the final day of the proliferation assay and subjected to quantitative PCR followed by liquid hybridization. There was an approximately 50% reduction of signal strength observed for IL-2 and IFN-γ mRNAs, whereas β-actin controls were not affected (Fig. 3). When B7 mRNA was assessed in IL-10 treated or untreated LC, there was no difference in signal strength (M. G. Lee and M. C. Udey, unpublished observations).

Differential effects of IL-10 on LC-dependent proliferation of Th1 and Th2 cells

To determine whether, in the proliferative response of T cells induced by IL-10–pretreated LC, there is a differential effect on Th1 and Th2 cells, the pigeon cytochrome c-specific Th1 clone AE7 and the conalbumin-specific Th2 clone D10.G4 were used to assess changes in the induction of thymidine incorporation by IL-10–treated LC compared with controls. Whereas LC-dependent proliferation of the Th2 clone D10.G4 was not affected by IL-10 treatment of LC with IL-10 before, or during, coculture with Th1 clone AE7 or with Th1 clone LNC.2 almost completely inhibited T cell proliferation when fresh or 1-day–cultured LC were used as APC (Fig. 4 and Table I). The effect of IL-10 was shown to be independent of Ag processing because the same changes were seen when the immunogenic cytochrome c fragment 81–104 was used as Ag (Fig. 5). Addition of IL-10 mAB during IL-10 treatment completely reversed the effect of IL-10 (data not shown).

Cultured LC are resistant to IL-10 effects

Because LC that are cultured for several days exhibit some characteristics distinct from those of freshly prepared LC, we assessed the ability of IL-10 to inhibit the Ag presenting ability of cultured LC. EC suspensions were enriched for LC by density-gradient centrifugation and used as APC in proliferation assays. Whereas LC cultured in the presence of IL-10 induced a markedly diminished AE7 proliferation, LC exposed to IL-10 after 2 days of culture were largely resistant to the effects of IL-10 (Fig. 6). These results suggest that IL-10 affects one or more costimulatory factors that are already present on cultured but not fresh LC and exclude a direct effect of IL-10 on Th1 cells.

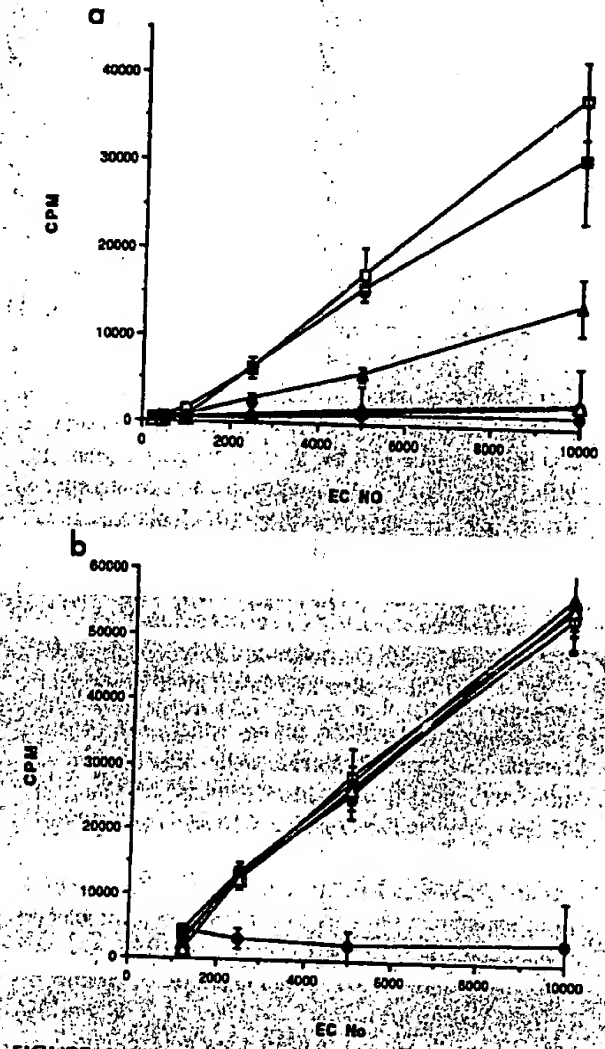

FIGURE 4. IL-10 pretreatment of LC inhibits the proliferation of Th1 (*a*) but does not affect the proliferation of Th2 (*b*) cells. LC were pretreated with 100 U/ml IL-10, mock COS supernatant, or medium for 1 day in the presence of Ag. These cells were then cocultured with AE7 (*a*) or D10.G4 (*b*) responder cells that were assessed for ^{3}[H]-TdR incorporation after 3 days. Results are from nontreated cells (*open squares*), mock COS supernatant treated cells (*closed squares*), untreated LC cocultured with AE7 in the presence of IL-10 (*closed triangles*), IL-10–pretreated LC cocultured in the presence of IL-10 (*open circles*), IL-10 pretreated LC cocultured in the absence of IL-10 (*open triangles*), and LC cocultured with AE7 in the absence of Ag (*closed circles*). *b*, Effect of IL-10 on the proliferation of Th2 cells. LC were pretreated as described under *a* but cocultured with D10.G4. Groups have the same assignments as under *a*.

Pretreatment with IL-10 abrogates the costimulatory capability of cultured BALB/c LC

To test the costimulatory abilities of IL-10–pretreated LC, BALB/c LC incapable of presenting Ag to our H-2^k restricted AE7 Th1 cell clone were used to restore proliferation when AE7 T cells were cocultured with fresh C3H LC in the presence of IL-10 (100 U/ml) and Ag. Whereas untreated cultured BALB/c LC restored the proliferative response of AE7 cells that were cultured with IL-10–pretreated C3H LC, IL-10–pretreated BALB/c LC did not provide the necessary costimulatory signals to cause T cell proliferation (Fig. 7). The data indicate that IL-10 negatively regulates a costimulatory signal on LC necessary for induction of TH1 cell proliferation.

Table I
IL-10 pretreatment of LC inhibits proliferation of the PPD-specific TH1 clone LNC.2[a]

EC No	Pretreatment of LC		
	Medium alone	Control-Sup	IL-10
50 000	52 420 ± 3319	51 092 ± 1584	18 072 ± 2180
25 000	29 765 ± 2537	29 576 ± 3037	8123 ± 296
10 000	12 736 ± 1384	10 501 ± 1298	1081 ± 61
5000	5038 ± 92	5032 ± 1798	956 ± 52

[a] Cell treatments and culture conditions were analogous to those described in the legend for Figure 4. The control supernatant was from mock-COS cells. Data are expressed as mean cpm ± SEM. Background values varied from 850 cpm for APC to 390 cpm for T cells.

IL-10 converts LC from immunogenic to tolerogenic APC

As the absence of costimulation in the presence of Ag should lead to T cell anergy or tolerance and as we assumed the IL-10 effects were mediated by the inhibition of expression of a costimulatory molecule on LC, we rescued T cells from cocultures with IL-10 or control-treated LC and restimulated them either immediately or after a rest period of 1 to 5 days as described. Whereas T cells cultured in the presence of Ag plus control supernatant or medium responded vigorously to restimulation with Ag-pulsed–untreated LC, T cells cultured with IL-10–pretreated LC were unresponsive to further stimulation (Fig. 8). IL-10–induced anergy was shown to be long lasting (at least 5 days; data not shown) and Ag-dependent because T cells pretreated with IL-10–treated LC in the absence of Ag responded normally to further stimulation. All T cells responded vigorously to stimulation with IL-2 (Fig. 8).

Discussion

It has been reported that IL-10 inhibits APC activity of macrophages and monocytes, whereas B cells and cells of the dendritic system were found to be resistant to the effects of IL-10 (3, 21). Because we previously identified murine keratinocytes as a source of IL-10 in primary immune responses in skin, we characterized the effects of IL-10 on LC accessory cell and APC function.

The data presented here demonstrate that IL-10 pretreatment of LC inhibits the induction of Ag-specific T cell proliferation in Th1 clones but has no effect on responses

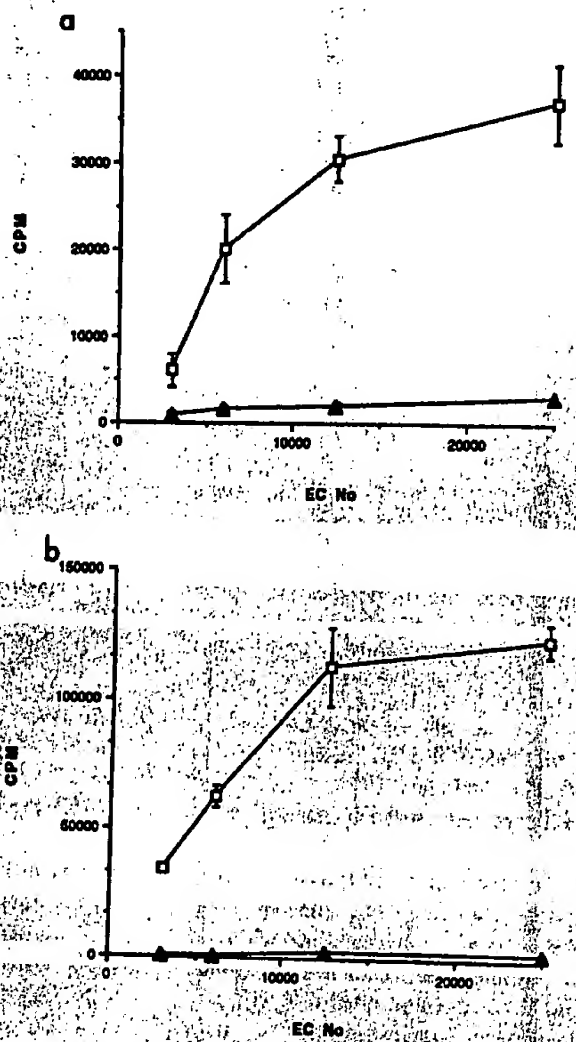

FIGURE 5. IL-10 pretreatment of LC inhibits protein-induced proliferation of Th1 cells. *a*, LC were pretreated with 100 U/ml IL-10 (*closed triangles*) or left untreated (*open squares*) for 1 day. Afterward, the IL-10–pretreated LC were cocultured with 2×10^4 AE7 cells in the presence of 100 U/ml of IL-10 plus 60 μg/ml whole cytochrome c, and the untreated LC were cocultured with 2×10^4 AE7 cells with cytochrome c in the absence of IL-10. *b*, Effect of IL-10 on peptide-induced proliferation of Th1 cells. Cells were treated as described under *a*, but instead of the whole cytochrome c, the immunogenic cytochrome c peptide 81–104 (kindly provided by Dr. J. Ashwell, NCI, Bethesda, MD) was added at 0.01 M.

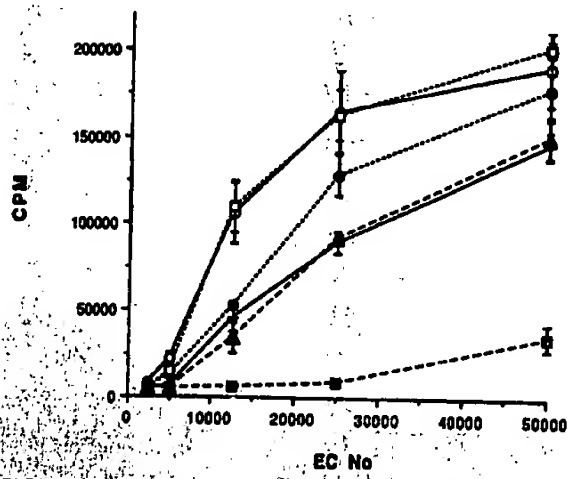

FIGURE 6. Two-day–cultured LC are less sensitive than are freshly prepared LC to the IL-10 inhibition of APC function. LC were cultured for 2 days with either the mock COS control supernatant, or were left untreated and then cocultured with AE7 responder cells (2×10^4) and cytochrome c in the presence or absence of IL-10 or the mock COS control supernatant. Groups include untreated LC cultured with T cells in the absence of IL-10 (*open circles*), mock supernatant-treated LC cultured with T cells in the presence of mock supernatant (*closed circles*), and untreated LC cultured with T cells in the presence of IL-10 (*open squares*). Freshly prepared LC cultured with T cells in the absence of IL-10 (*closed triangles*), freshly prepared LC cultured with T cells in the presence of mock supernatant (*crosses*) and freshly prepared LC treated with IL-10 (*closed squares; dashed line*) were also tested at the same time using the same responder cells.

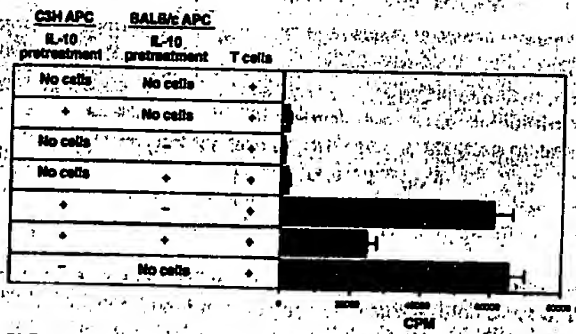

FIGURE 7. Untreated BALB/c LC can provide costimulatory signal(s) to induce T cell proliferation. Cocultures included IL-10 treated (+) or untreated (−) freshly prepared C3H LC (or no C3H LC at all), AE7 cells and whole cytochrome c, as well as untreated (−) or IL-10 pretreated (+) (for 3 days) BALB/c LC (or no BALB/c LC at all).

of Th2 clones. Further, Th1 cells that were incubated with IL-10–pretreated LC and Ag were unresponsive to further stimulation by nontreated Ag-pulsed LC. This anergy persisted for at least 5 days after the first incubation. However, anergic Th1 cells remained responsive to IL-2.

It has been the effect of IL-10 on the expression of MHC class II molecules on monocytes that was thought to be mainly responsible for the influence of this cytokine on Ag presentation (10). However, downregulation of MHC class II alone insufficiently explains the inhibitory effects of IL-10 on macrophage/monocyte-induced T cell proliferation. Recently, de Waal-Malefyt and coworkers (10) observed that although IL-4 reversed the IL-10 effects on MHC class II expression, IL-4 did not reverse the

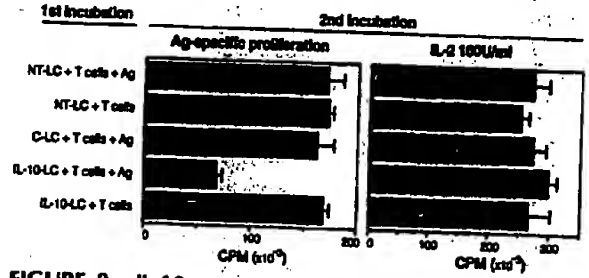

FIGURE 8. IL-10 treatment renders LC tolerogenic. LC were cultured overnight either in the presence of 100 U/ml IL-10 (IL-10-LC), COS-mock supernatant (C-LC), or with medium alone (NT-LC). Afterward, EC suspensions were enriched for LC that were then precultured in 24-well plates with AE7 cells with or without Ag as indicated (1st incubation). After 16 to 20 h, AE7 cells were rescued and recultured immediately with fresh LC in the presence of Ag (2nd incubation). The *left bar graph* shows the Ag-specific response, the *right bar graph* shows the T cell response to 100 U/ml IL-2. Further, T cells precultured in 100 U/ml IL-10 in the absence of LC were fully responsive to subsequent stimulation by LC plus Ag in the absence of IL-10. Background counts for nonrestimulated AE7 cells were 35 000 cpm. Identical results were obtained when the AE7 cells were recultured after resting for 1 day in complete medium containing 2 U IL-2/ml (data not shown).

macrophage-mediated suppressive effects of IL-10 on T cell proliferation.

The data presented here suggest that IL-10 influences the Ag-presenting functions of epidermal LC by modulating costimulatory molecules on the surface of freshly prepared LC. Ample evidence exists that cultured LC (that are resistant to the effects of IL-10) already express all necessary costimulatory molecules on their surfaces, whereas fresh LC are immature in this respect (22). Therefore, it may be that IL-10 treatment of fresh LC prevents an upregulation of such a costimulator thereby altering LC in such a way as to make them tolerogenic (23, 24). In the costimulation assay, nontreated allogeneic LC provided the requisite costimulatory molecules. A potential candidate molecule for the effects of IL-10 on LC is B7/BB1 whose expression is reported to be upregulated on cultured LC, at least in humans (25). A lack of B7 expression is associated with clonal anergy in various systems (22). However, using a reverse transcriptase-PCR technique, we could not detect any downregulation of B7 mRNA with IL-10 treatment of LC. Our data are in accord with a report by Ding and Shevach (21) wherein IL-10 failed to inhibit Ag-presenting functions of peripheral blood dendritic cells when these cells were used in an anti-CD3 proliferation assay using peripheral $CD4^+$ T cells as responders. Blood dendritic cells are more similar to cultured LC and therefore probably already contain all necessary costimulatory factors. This could potentially make them unresponsive to the effects of IL-10 and may explain the negative data reported by these investigators.

The finding that IL-10 is produced by cells within the epidermis and exerts significant influences on LC has important implications. IL-10 is induced late in the cytokine cascade initiated during the generation of primary immune responses in skin. From our in vitro studies and those of others (3), we predict that IL-10 needs at least 6 to 8 h to affect its target cells and suggest that IL-10 exerts counterregulatory effects late in inflammatory processes, e.g., by converting LC migrating to regional lymph nodes at later phases of the inflammatory process to specifically tolerizing cells thereby potentially limiting the number of specifically sensitized T cells generated. In addition, IL-10 might indirectly facilitate the outgrowth of Th2 cells by abrogating IFN-γ and IL-2 production of Th1 cells thereby downregulating delayed-type hypersensitivity reactions and explaining earlier results from our laboratory that LC preferentially promote outgrowth of Th2 clones in vitro. In another system, Simon et al. (19) demonstrated that UV-B-treated LC were capable of anergizing $CD4^+$ Th1 cell clones. These authors also demonstrated that UV-treated LC were fully active in inducing Th2 cell proliferation. Although Simon et al. could not detect a soluble inhibitor in their system, it has been shown that UV-treated keratinocytes are capable of producing biologically active IL-10 (26). Thus, the anergizing effects of UV light may be mediated via release of IL-10 by keratinocytes. This possibility and the precise mechanism of IL-10 action on LC, as well as its in vivo functional effects, are worthy of further pursuit.

Acknowledgments

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Dendritic cells as a tool to induce anergic and regulatory T cells

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The induction of antigen-specific T-cell tolerance in the thymus and its maintenance in the periphery is crucial for the prevention of autoimmunity. As well as their stimulatory functions, there is growing evidence that dendritic cells, acting as professional antigen-presenting cells, also maintain and regulate T-cell tolerance in the periphery. This control function is exerted by certain maturation stages and subsets of different ontogeny, and can be influenced by immunomodulatory agents. What is the current state of knowledge of the 'immunoregulatory' properties of dendritic cells and how might tolerance-inducing dendritic cells be relevant to therapeutic applications in humans?

T cells with the capacity to respond to organ-specific autoantigens (autoAgs) and thereby produce harmful autoimmune lesions are present in the normal immune repertoire[1,2]. However, one of the hallmarks of our immune system is its ability to protect us from an abundance of potentially pathogenic microorganisms but avoid pathological reactivity with self constituents. This avoidance is called self-tolerance and the failure of immunological self-tolerance can lead to autoimmune disease[1,3]. Although the etiology of autoimmune disease is at present largely unknown, self-reactive T cells are considered to be key mediators of many autoimmune diseases[4]. However, there is increasing evidence that T cells also exert regulatory functions, thereby preventing or limiting autoimmune diseases[5]. The existence of such anergic or regulatory T cells (T_{reg} cells) has been of great interest to immunologists for some time, but only recently has it become clear that certain subtypes of dendritic cell (DC) play an essential role in inducing and modulating these T cells both *in vitro* and *in vivo*[6].

DCs are a family of professional Ag-presenting cells (APCs) that are present in trace amounts in virtually all organs. The ability of DCs to process and present various types of Ag is unmatched in the human body[7]. The major function of DCs has been defined and various subpopulations identified (e.g. lymphoid or myeloid, DC1 or DC2 and splenic or epidermal DCs); a general property of all subtypes of DC seems to be that they pass through several levels of maturation during their life-span[8]. Immature DCs express low levels of MHC class II and costimulatory molecules, but the surface expression of these molecules is dramatically upregulated during maturation in response to appropriate inflammatory stimuli[8,9]. Additionally, terminally differentiated DCs express specific maturation markers, such as 33D1 in the murine system[10] or CD83 on human mature DCs (Ref. 11). Functionally, immature DCs in the periphery are specialized for Ag capture by endocytosis or macropinocytosis, whereas maturating DCs lose these capacities but, in parallel, significantly enhance their stimulatory properties for naive $CD4^+$ and $CD8^+$ T cells[9]. In contrast to these immunostimulatory properties, there is expanding evidence that certain subpopulations of DC, such as liver-derived DCs (Refs 12,13), interleukin-10 (IL-10)-modulated DCs (Ref. 14) or $CD8\alpha^+$ lymphoid-derived DCs (Ref. 15), are able to downregulate immune responses. These different DC subpopulations all represent a more or less immature phenotype. It is the aim of this review to summarize the current evidence that this immature DC phenotype is characteristic of the 'regulatory' DCs that might act as guardians for the induction and maintenance of peripheral T-cell tolerance and the prevention of pathological autoimmune reactions.

The induction of tolerizing DCs by IL-10

Originally, IL-10 was identified as a cytokine-synthesis-inhibiting factor (CSIF), particularly with regard to the suppression of interferon γ (IFN-γ) production by T helper 1 (Th1) cells. Subsequently, it was found that this cytokine suppresses multiple activities of the immune response[16,17]. The immunosuppressive properties of IL-10 on DCs are caused by a reduction in the upregulation of expression of MHC class II molecules and several costimulatory and adhesion molecules and, in the human system, also the DC-specific marker CD83 (Refs 14,18–20). Additionally, analysis of supernatants from IL-10-treated human DC cultures demonstrated an inhibited production of inflammatory cytokines [e.g. IL-1β, IL-6 and tumor necrosis factor α (TNF-α)] and a lack of IL-12 synthesis[21–26]. These altered properties were only seen if IL-10 was added to immature (IL-10-sensitive) DCs, indicating that IL-10 modulates the function of immature DCs and inhibits their terminal differentiation; mature DCs are resistant to IL-10 (Refs 14,27).

It has been demonstrated that freshly enriched murine Langerhans cell (LC) suspensions (immature skin DCs) pretreated with IL-10 inhibited the induction of Ag-specific proliferation of Th1 clones but had no effect on the responses of Th2 clones[28]. Also, IL-10 inhibits tumor-Ag presentation by murine epidermal LCs (Refs 29–31). Furthermore, human IL-10-modulated DCs from peripheral blood induce an alloAg-specific anergy of

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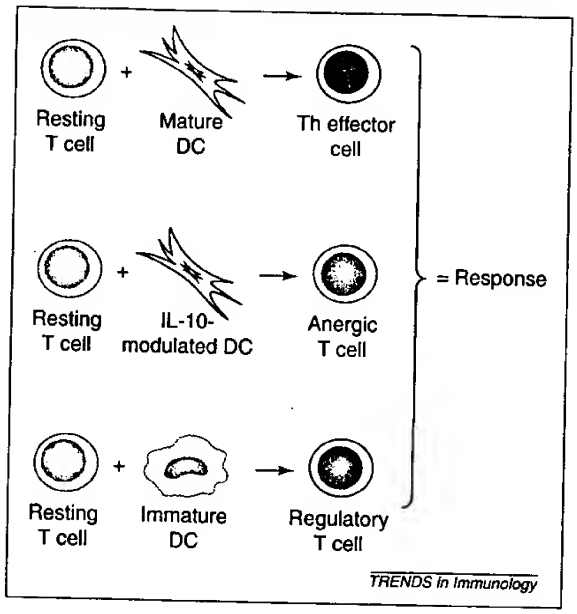

Fig. 1. A model of T-cell differentiation depending on dendritic cell (DC) maturation and modulation. The activation of resting CD4+ T cells by different DC populations leads to the development of distinct T-cell subsets. Mature DCs, acting as accessory cells, induce the development of classical T helper (Th) effector cells. The presence of interleukin-10 (IL-10) during the maturation of DCs results in a shift in DC phenotype. IL-10-modulated DCs induce anergic T cells, characterized by inhibited antigen-specific proliferation, a profoundly reduced production of IL-2 and interferon γ (IFN-γ), and a downregulated expression of CD25. Finally, repetitive stimulation with immature DCs – in the absence of inflammatory stimuli – induces the development of regulatory T cells (T_{reg} cells) that suppress the activation of T effector cells, independent of their antigen specificity.

alloreactive CD4+ and CD8+ T cells and an Ag-specific anergy in hemagglutinin or melanoma Ag-specific CD4+ and CD8+ T cells[14,27]. This state of anergy was characterized by an inhibited T-cell proliferation and reduced production of IL-2 and IFN-γ. The induction of anergy requires direct cell–cell contact between T cells and DCs as well as soluble factors produced by IL-10-treated DCs. In contrast to T_{reg} cells, anergic T cells induced by IL-10-treated DCs are characterized by a markedly reduced expression of the IL-2 receptor α-chain (CD25), and anergic T cells produce no immunomodulatory cytokines, such as IL-10 or transforming growth factor β (TGF-β)[14,27]. Pretreatment of T cells with IL-10-modulated DCs also affected the effector functions of the T cells. It was shown that, in contrast to optimally stimulated CD8+ T cells, anergic melanoma peptide-specific CD8+ T cells failed to lyse tumor cells[27]. In addition, comparisons of human DCs isolated from responding or progressing melanoma metastases demonstrated markedly increased production of IL-10 in the tumor cells of progressively growing metastases[32]. Furthermore, DCs derived from the progressive tumors, but not from the responding metastases, induced a state of Ag-specific anergy in cocultured T cells[32]. As a consequence, this finding supports earlier data showing that the production of IL-10 by tumor cells and/or tumor-infiltrating ymphocytes might serve as a mechanism for tumor-induced anergy.

The production of IL-10 and/or other immunosuppressive cytokines, such as TGF-β, which modulate the functional activities of DCs, might be a way of limiting harmful, autoreactive T-cell responses by converting immature DCs into 'tolerizing' APCs. However, the release of IL-10 in tumors might deactivate tumor-specific immune responses and serve as a mechanism for immune escape.

The induction of regulatory T cells by immature DCs

T_{reg} cells have been extensively characterized in the murine system as a subpopulation of 5–10% of all peripheral CD4+ T cells[33]. When freshly isolated, these cells express CD25 and intracellular cytotoxic T-lymphocyte antigen 4 (CTLA-4), do not proliferate after activation and suppress effector T cells in an Ag-nonspecific fashion[34]. The molecules involved in this cell contact-dependent suppressive effect are largely unknown. The development of T_{reg} cells from mice thymi occurs ≈3 days postpartum, and their maintenance possibly depends on the recognition of autoAgs in the periphery[35], in combination with the triggering of CD28 and CD154 coreceptors[36,37]. Thus, it has been suggested that the thymus has a third function, in addition to the negative and positive selection of T cells, namely the generation of T_{reg} cells[38]. Eradication of T_{reg} cells in mice results in the development of various autoimmune diseases, such as gastritis and thyroiditis[39]. The transfer of CD25-depleted CD4+ T cells resulted in autoimmune reactions against target organs, indicating that the main function of the CD25+ T_{reg} cells is the suppression of pathological autoaggressive T-cell responses[40,41]. The suppressive mechanisms by which CD25+ T_{reg} cells exert their inhibition *in vitro* were found to be dependent on a direct T_{reg}–T effector cell contact[34,42]. The Ag specificity of T_{reg} cells is still largely unknown. However, it has been shown recently in a T-cell receptor transgenic mouse model that influenza-peptide-specific T_{reg} cells develop after the expression of the peptide in these mice[43]. However, it remains to be confirmed whether T_{reg} cells with specificity for defined (auto)Ags also exist in nontransgenic mice and humans.

Regulatory T cells consist of various subtypes

With regard to accessory cells, experimental evidence points to the fact that immature DCs can mediate tolerance, presumably by the induction of T_{reg} cells[44,45] (Fig. 1). Recently, it has been shown in humans that immature DCs *in vitro* can induce alloAg-reactive T_{reg} cells[6]. Repetitive stimulation of naive CD4+ T cells with allogeneic immature DCs, in the absence of immunomodulating agents, resulted in the differentiation of naive T cells into nonexpanding CD4+CD25+ T-cell populations. These T-cell populations induced by immature DCs *in vitro* produce high levels of IL-10, but no IL-4, IL-5 or IL-2, and they are poorly proliferative after stimulation[6]. The IL-10-producing T_{reg} cells can act directly on activated Th1 cells and inhibit their Ag-specific proliferation and cytokine production in a cell contact-dependent manner. The suppressive activity of these T_{reg} cells – in contrast to the anergic T cells induced by IL-10-modulated DCs – is Ag-nonspecific and can be partially inhibited by the addition of exogenous IL-2 (Ref. 6). Thus, the functional activities of human T_{reg} cells induced *in vitro* are very similar to the described properties of the murine CD4+CD25+ T_{reg} cells *ex vivo*.

Table 1. The features of regulatory and anergic T cells induced by DCs[a]

	Induction by:	
	Immature DCs	IL-10-modulated DCs
Type of T cell induced	Regulatory T cells	Anergic T cells
Proliferative capacity	Low	Low
Cytokine profile	IL-10^{++}, IL-2^{-}, IFN-γ^{-} and IL-4^{-}	IL-10^{-}, IL-2$^{+/-}$, IFN-$\gamma^{+/-}$ and IL-4^{-}
Suppressive activity	Antigen-nonspecific	Antigen-specific
Phenotype	CD4^{+}	CD4^{+} or CD8^{+}

[a]Abbreviations: DC, dendritic cell; IFN-γ, interferon γ; IL, interleukin.

The fact that IL-10 synthesis by T_{reg} cells induced by immature DCs requires repetitive restimulation *in vitro* favors the possibility that CD4^{+}CD25^{+} T cells derived from the thymus might represent a 'resting state' of T_{reg} cells, which possibly differentiate into IL-10-producing T_{reg} cells after chronic stimulation. Interestingly, immature DCs do not only suppress the inflammatory activities of Th1 cells *in vitro*. In a recent publication, it was demonstrated that the injection of influenza matrix peptide-pulsed immature DCs led to an inhibition of the Ag-specific effector functions of CD8^{+} T cells *in vivo*[46]. Furthermore, it was shown that matrix peptide-specific, IL-10-producing T cells were induced simultaneously. Following immunization with immature DCs, increased numbers of Ag-specific T cells were detectable by tetramer staining. However, these T cells lacked killer activity and had reduced IFN-γ production[46].

Another subset of IL-10-producing human T_{reg} cells, termed Tr1 cells, could be induced by repetitive activation of human T cells with alloAgs in the presence of IL-10 (Refs 47,48). Tr1 cells produce large quantities of IL-10, as well as moderate levels of TGF-β, IFN-γ and IL-5. In contrast to the T_{reg} cells induced by immature DCs *in vitro*, the suppressive activity of Tr1 cells is partially dependent on the production of the immunomodulating cytokines IL-10 and TGF-β. Furthermore, it has been shown very recently that IFN-α strongly upregulates the expression of CD40, CD80, CD83 and CD86 in human peripheral CD11c^{+} DCs and that these DCs induced the development of IL-10-producing alloreactive CD4^{+} T cells *in vitro*. Collectively, these data suggest that different regulatory T-cell populations develop under the influence of varying conditions. In addition, the regulatory properties of these distinct T-cell subsets are obviously based on diverse suppressive mechanisms.

Professional APCs are required for the induction of regulatory T cells

The capacity of DCs to induce stimulatory T cells versus T_{reg} cells is strictly dependent on the state of DC maturation[6] (Table 1). In the absence of inflammatory stimuli, immature, resting DCs are relatively poor stimulators of the proliferation of resting T cells and can presumably induce tolerance. These downregulatory properties of immature DCs can be enhanced by immunomodulating growth factors, such as TGF-β, prostaglandin and IL-10 (Refs 14,49). Therefore, our current knowledge suggests that the induction of tolerance versus immunity in the periphery could be determined by the ratio of resting/immature DCs to activated/mature DCs (Refs 6,50). T_{reg} cells could be induced by immature DCs in the context of autoAgs and subsequently, their phenotype and function could be maintained in the periphery through interactions with autoAg-presenting mature/activated DCs. In murine transplantation studies, immature DCs can mediate tolerance, presumably by the induction of such T_{reg} cells[44]. Our own data, as well as a report by Dhodapkar *et al.*, demonstrated that immature DCs, *in vitro* as well as *in vivo*, induce the development of T_{reg} cells. Regarding the maintenance of T_{reg} cells in the periphery, there is good evidence that the long-lasting function of T_{reg} cells depends on the presence of the respective autoAgs (Ref. 35). Moreover, it has been found that the homeostasis of T_{reg} cells depends on cosignaling through CD154 and CD28 (Refs 36,37). Thus, these data suggest that the development of T_{reg} cells is certainly not simply the result of insufficient coactivation by nonprofessional APCs but is dependent on professional APCs that express CD40 and CD80/CD86. These ligands for CD154 and CD28 in the periphery are – in the absence of inflammation – only present on DCs and not on macrophages, B cells or epithelial cells. Therefore, it has been suggested that DCs control peripheral T-cell tolerance[51]. According to this hypothesis, immature DCs, in the absence of inflammatory signals, take up protein Ags in the periphery from apoptotic cells[45], enter the regional lymph nodes and prime T-cell precursors to become regulatory rather than effector T cells[45,51]. Primed T_{reg} cells home to the tissues and, in inflammatory situations, will be reactivated by autoAg-presenting DCs to downregulate the effector T-cell responses at the site of inflammation[51].

Both types of DC (immature and IL-10-modulated DCs) show similarities as well as differences (Table 2). Whereas IL-10-modulated DCs are able to inhibit the proliferation and cytokine production of differentiated T cells[14,27], the stimulation of effector T cells with immature DCs induced a normal T-cell proliferation and cytokine profile[6]. The influence of immature DCs is restricted to resting/naive T cells and the induction of T_{reg} cells is dependent on repetitive stimulation by immature DCs (Ref. 6). By contrast, IL-10-modulated DCs induce Ag-specific anergy even after a single contact with the T effector cell. These data suggest that the two DC populations have different functions in the downregulation of T-cell responses: a direct Ag-specific suppression of effector T cells by IL-10-modulated DCs versus the indirect inhibition of effector T cells in an Ag-nonspecific manner through the activation of T_{reg} cells by immature DCs.

Lymphoid-derived DCs in tolerance induction

Apart from immature DCs, another subtype of DC (CD8α homodimer-expressing lymphoid-derived

Table 2. The similarities and differences between immature DCs and IL-10-modulated DCs[a]

Immature DCs	IL-10-modulated DCs
Weak expression of MHC and costimulatory molecules[6]	Weak expression of MHC and costimulatory molecules[14]
Maturation inducible by inflammatory stimuli[6,45]	Maturation resistant[14,27]
Present in all peripheral, noninflammatory tissues[8]	Present in IL-10-producing tumors[32] and UV-irradiated skin[65]
Involved in the induction of regulatory T cells[6], no direct influence on effector T cells[6]	Direct suppressive effect on effector T cells[14,27]

[a]Abbreviations: DC, dendritic cell; IL-10, interleukin-10; UV, ultraviolet.

DCs) has been implicated in tolerance induction[52]. The functional properties of lymphoid-derived DCs (LDCs) were originally described by Ardavin *et al.* using highly purified CD8α-expressing cells[53]. LDCs apparently originate from precursors in the thymus and are located in the thymic medulla and T-cell zones of the spleen and lymph nodes[52,53]. They share several properties with 'classic' DCs, including dendritic morphology and the expression of surface molecules required for the stimulation of naive T cells, such as MHC class I and II Ags, CD80, CD86 and CD54. LDCs have so far only been described in the mouse [although some investigators feel that the $CD4^+$IL-3 receptor (IL-3R)$^+$ DCs represent their human equivalents[54]] and the major phenotypic marker that distinguishes them from conventional DCs is CD8α. Additionally, murine LDCs do not express 33D1 (Ref. 55), but do express high levels of the Ag receptor DEC-205 (Ref. 56). In cell culture, the growth of LDCs is largely dependent on IL-3, whereas myeloid-derived DCs depend on granulocyte–macrophage colony-stimulating factor (GM-CSF)[57]. LDCs seem to be unable to process complex protein Ags. When loaded with the appropriate peptide or superAg, however, these cells will stimulate potent T-cell responses. Their inability to process protein Ags, together with the discovery that the majority of spleen DCs are LDCs and that only LDCs are located in the T-cell zones of secondary lymphoid organs in nonimmunized mice (myeloid-derived DCs only migrate to these areas following activation by Ags), makes LDCs ideal, candidate APCs for tolerance induction[58,59]. Some investigators have demonstrated that LDCs delete mature T cells by a CD95-dependent, Ag-specific mechanism[60]. This effect is however, debated by others[61]. It should be noted at this point that the evidence that links LDCs to tolerance induction is mainly circumstantial and no definite experimental data exist to support this notion. In addition, recent publications have discussed the controversial origin of LDCs, with some reports suggesting that myeloid-derived DCs and LDCs originate from the same precursor[61–64].

In conclusion, future studies might indeed reveal different ontological origins for subsets of DCs. Whether certain subtypes of DC will indeed prove to be 'specialized' in tolerance induction or whether function is dependent on the activation and maturation status of any DC subtype remains to be seen.

In vivo relevance

The prevention of T-cell sensitization through modulation by professional APCs is a well-known phenomenon. One of the best-studied models is the altered APC function after ultraviolet (UV) irradiation of the skin. UV radiation has both local and systemic effects. Locally, it induces the production of immunosuppressive cytokines and an alteration of the Ag-presenting function of epidermal LCs. Systemic suppression results when suppressive T cells are induced by altered LCs and the action of immunosuppressive cytokines, such as IL-10 (Refs 65,66). As the systemic immunsuppressive effect can be transferred from animal to animal by T cells the presence of 'suppressor' (regulatory?) T cells is probable.

Regulatory T cells in tumors

Additionally, immature, 'tolerogenic' DCs have been described in tumors, such as melanoma and breast cancer[32,67,68]. Tumor-associated DCs usually have a low allostimulatory capacity and represent an immature phenotype. In malignant melanoma, this phenotype was linked to the presence of IL-10 in progressing metastases[32]. When DCs were isolated from the melanoma lesions of patients with progressing and regressing metastases, marked functional differences were observed. Whereas the function of DCs isolated from regressing lesions was normal in terms of their capacity to induce allogeneic T-cell proliferation, DCs from progressing lesions were reduced in their ability to support allogeneic T-cell responses. Phenotypic analysis revealed that DCs from progressing lesions showed decreased expression of CD86 and CD83 as compared with DCs from regressing metastases. An analysis of cytokine release by the tumor tissue showed that progressing lesions produced high levels of IL-10, whereas regressing lesions produced IL-2 and IL-12, but virtually no IL-10. Supernatants from cell cultures of progressing lesions were able to suppress allogeneic T-cell responses, an effect that was neutralized by the addition of anti-IL-10 monoclonal antibody (mAb). Furthermore, DCs from progressing lesions induced anergy in anti-CD3 Ab-stimulated $CD4^+$ T cells[32].

These results were confirmed for breast cancer[69,70]. Here, immature $CD1a^+$ DCs are retained in the tumor bed in >90% of all samples, whereas mature, $CD83^+$ DCs are confined to peritumoral areas. Thus, immature DCs seem to be found preferentially within tumors and might be used to divert immune reactions towards the induction of anergy or regulation, representing a means of immune escape for the tumor (Fig. 2).

These findings are further supported by *in vivo* injection studies comparing immature DCs with

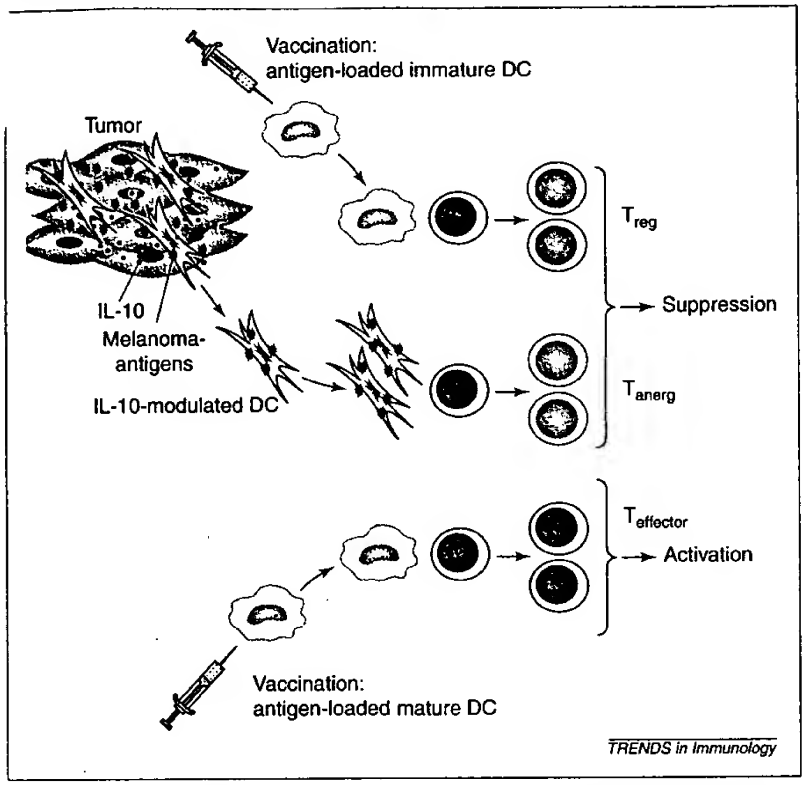

Fig. 2. A model for the induction of tumor antigen-specific tolerance by dendritic cells (DCs) *in vivo*. Tumors, such as melanomas[32] or breast cancer[69], are able to produce large amounts of interleukin-10 (IL-10), which possibly modulates the function of tumor-associated DCs. This assumption is supported by data showing that IL-10-modulated DCs that present tumor-associated antigens in peripheral lymph nodes induce anergic T (T_{anerg}) cells rather than effector T ($T_{effector}$) cells[27]. However, experimental and clinical data have shown the usefulness of antigen-pulsed DCs as adjuvants for the immunotherapy of tumor patients. Expanding evidence indicates that terminally differentiated, mature DCs should be prioritized for use in the induction of $T_{effector}$ cells by vaccination protocols. Antigen-loaded immature DCs should not be used for vaccination because of the risk of inducing regulatory T (T_{reg}) cells that would further enhance the tumor-specific tolerance[6].

mature DCs in melanoma patients[71]. Whereas mature DCs reproducibly induced strong T-cell responses, negligible responses were observed using Ag-loaded immature DCs within the same patient. Furthermore, as it has been demonstrated that immature DCs might also induce T_{reg} cells and Ag-specific tolerance *in vivo*[72], special care needs to be taken in vaccination protocols using DCs. For tumor patients, the injection of immature, IL-10-sensitive DCs might in fact cause the Ag-specific downregulation of T-cell immune responses against the vaccinated tumor Ag (Fig. 2).

Regulatory T cells in liver transplantation and autoimmune diseases

As well as the studies performed with tumors, the first evidence that certain subtypes of DC are involved in tolerance induction *in vivo* stems from liver transplant studies[13]. The APCs that are thought to mediate the immune privilege of liver allografts are immature DCs. It is known from transplant studies that donor interstitial DCs migrate to host secondary lymphoid tissue following organ transplantation, where they interact with specific, donor-reactive T cells. Thomson *et al.* have shown that immature myeloid-derived DCs propagated from normal murine liver are deficient in costimulatory molecules. They migrate *in vivo* to the T-cell areas of secondary lymphoid tissue, where they persist for weeks in allogeneic recipients[12,13]. These liver-derived DC progenitors can prolong allograft survival. *In vitro*, liver-derived DCs induce only weak proliferative or cytotoxic responses in allogeneic T cells compared with mature, bone marrow-derived DCs. These liver-derived DCs do not induce IFN-γ release by T cells, but are prone to induce the release of IL-10. Also, liver-derived DCs have been shown to produce IL-10 themselves. Taken together, these data indicate that immature liver-derived DCs regulate immune responses by the induction of some kind of T_{reg} cell[12,13]. In agreement with the data from liver transplant studies, it has been known for some time that the DCs in peripheral organs are usually of the immature phenotype (e.g. LCs in the epidermis). Also, the majority of DCs present in the lymph nodes have been described as representing an immature phenotype[72,73]. Because naive T cells can be found in peripheral tissues it might be possible that contact with self-Ag-presenting immature DCs in the periphery or lymphoid organs constantly generates and maintains T_{reg} cells that prevent the induction of autoimmunity. A more appropriate term for this mechanism might be crossregulation rather than crosstolerance[45]. Only when DCs are properly activated by inflammatory stimuli, such as bacterial particles, DNA-motifs or cytokines, will they be able to induce the activation and differentiation of effector T cells. This might also explain the frequent finding that autoimmune diseases are first diagnosed following common infections.

Therapeutically, the induction of T_{reg} cells by immature DCs could be useful in patients with autoimmune diseases. As the regulatory properties of the T cells generated are Ag-independent, it would be feasible to utilize these T cells in human autoimmune diseases with unknown target Ags. Because the T_{reg} cells are induced by immature DCs in an autologous situation, clinical trials are not hampered by too many ethical restrictions. The first trials with murine $CD4^+CD25^+$ T_{reg} cells isolated from the lymph nodes or blood in murine autoimmune models have been rather successful. Also, the injection of allogeneic immature DCs induces alloAg-specific T-cell tolerance *in vivo* and promotes unresponsiveness to skin or heart allografts. The generation of human DCs from peripheral progenitors and their modification by IL-10 might be the first step in the development of a treatment for patients with autoimmune or allergic diseases with known (auto)Ags. In this scenario, DCs generated from the patients might be deactivated by IL-10 and then reinfused to switch off a harmful immune reaction. Therefore, T_{reg} cells generated by immature or IL-10-modulated DCs might prove to be useful tools for treating many different human autoimmune and allergic diseases.

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Ig heavy-chain gene revision: leaping towards autoimmunity

Kimberly D. Klonowski and Marc Monestier

B cells can revise their antigen receptors outside the confines of the bone marrow by secondary Ig gene rearrangements. Although the initial motivation to perform these revisions might be to silence a self-reactive specificity, those B cells that reinitiate the recombination process can perform a series of 'leaping' rearrangements and inadvertently shift their receptor specificity towards autoimmunity. Heavy-chain receptor revision, coupled with other atypical rearrangements, might contribute to autoantibody production in systemic lupus erythematosus.

The rearrangement of Ig genes allows B cells to respond to the wide spectrum of foreign antigens (Ags) encountered over an organism's lifetime. The combination of heavy-chain variable, diversity and joining (V_H, D and J_H) genes chosen to shape the repertoire represents the first mechanism by which developing B cells can generate this diversity. V(D)J rearrangement occurs in highly regulated steps, during which gene segments are cut at the recombination signal sequences (RSS) flanking each Ig gene by the enzymes encoded by recombinase-activating gene 1 (*RAG1*) and *RAG2*. This process is initiated when pro-B cells in the bone marrow recombine a D gene with a downstream J_H gene to form a DJ_H complex. Next, the preformed DJ_H rearrangement combines with an upstream V_H gene, generating the complete heavy-chain variable region gene. A similar procedure occurs for the light-chain locus at the later pre-B-cell stage, with the recombination of V_L and J_L genes. Once productively rearranged, heavy and light chains pair to form the complete antibody (Ab) molecule, and the immature B cell proceeds through selection checkpoints and exits the bone marrow to join the peripheral lymphocyte pool[1].

B cells producing Ab molecules that recognize self Ags are subject to inactivation through various mechanisms of tolerance. The B cell might undergo clonal deletion or simply become Ag-unresponsive or anergic[2,3]. A further possibility is that the B-cell Ag receptor is edited by a continuing or secondary rearrangement of Ig genes[4,5]. When used as a mechanism of tolerance, receptor editing is more economical than deletion or anergy because these autoreactive B cells can lose self-reactivity but remain viable and therefore contribute to mounting an effective immune response. Receptor editing takes place centrally in the bone marrow, and a similar process, termed receptor revision, can occur in mature B cells in the periphery[6,7]. Receptor revision is not unique to B cells; recent observations suggest that mature CD4+ T cells in the periphery can revise the β chain of the T-cell receptor[8]. In contrast to B-cell receptor (BCR) editing in the bone marrow, which is tolerance-driven, the stimulus to perform BCR revision in the periphery is unclear. Although it might still contribute to the maintenance of tolerance, receptor revision in peripheral sites could also participate in shaping the Ab repertoire. For instance, several researchers have suggested that peripheral revision might allow B cells in germinal centers to escape apoptotic death by creating a higher affinity for the Ag (Refs 9,10). Such a situation is however, improbable as secondary rearrangement is a drastic process that is unlikely to increase the affinity for the

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ELSEVIER

Adjusting Immunosuppr ssion to the Identification of T-Cell Activating Mediators in R j cting Transplants: A Novel Approach to Rejection Diagnosis and Treatment

J. Strehlau, W. Maslinski, D. Chae, M. Pavlakis, J.H.H. Ehrich, and T.B. Strom

ACUTE renal allograft rejection is orchestrated by a T-cell-dependent sequence of immune events aimed to generate alloreactive and cytotoxic T-cell clones that promote graft destruction. Activated CD4+ T-helper cells play a decisive role in the regulation of this antiallograft response, and most immunosuppressive regimens utilize drugs interfering with cell activation (interleukin-2R-Ab, OKT3, CTLA-4-Fc), gene transcription (steroids, cyclosporine, tacrolimus), and proliferation (azathioprine, mycophenolate mofetil) of lymphoid cells.

The number of immunosuppressive drugs used for organ transplantation is steadily increasing. "Immuno-suppressive drug tailoring" has been advocated instead of the "one drug for all" or "random assignment" approach. However, currently now widely accepted tools are available to monitor the patients immune response and to choose appropriate drug regimens for prevention or treatment of rejection in a given patient.

Our group[1] and collaborators[2] have demonstrated that quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) is a both sensitive and specific tool to identify mediators of immune activation in rejecting renal allografts. Transplants undergoing rejection with cyclosporine (CyA)-based regimens did not exhibit transcripts of interleukin (IL)-2 but developed a significantly heightened gene expression of the alternative T-cell growth factors IL-7 and IL-15, RANTES, IL-10, and CTL effectors.[1,2] More recently we were able to show dynamics of gene transcription in children undergoing rejection and successful treatments.[3]

The specific pattern of posttransplant gene expression may be dependent on the organ transplanted, preservation injury; APC-T-cell interactions due to HLA differences; and costimulatory factors, time, genetics, and the drugs used to suppress this response. We have started to evaluate the differential effect of drugs on the generation of mediators identified in rejecting grafts (IL-2, IL-7, IL-15, CTLA-4, granzyme B [GB], perforin [P], Fas ligand [FL]) utilizing RT-PCR in IL-2, IL-7, and IL-15 induced activation of peripheral blood mononuclear cells (PBMC).

MATERIALS AND METHODS

We investigated the inhibitory effect of CyA, dexamethasone (Dex), and rapamycin (Rapa) on the proliferative response and gene expression of Ficoll-Paque isolated PBMCs from healthy adult blood donor volunteers (n = 8). Cells were cultured for 24 hours at 37°C in RPM1640 medium and prestimulated with 0.1 μg/mL PHA for 24 hours to induce high affinity cytokine receptors and washed thereafter. Drugs were added at desired blood level concentrations (CyA 100 ng/mL, Rapa 15 ng/mL) to 200,000 cells each 30 minutes prior to stimulation in 96-well microliter plates. Cells were stimulated by IL-2 (5 or 50 U/mL), recombinant IL-7 (5 U/mL) or IL-15-FLAG (5 U/mL, fusion protein sequence cloned by W. Maslinski). Proliferation was analyzed at 7, 24, 48, and 72 hours by 3H-thymidine incorporation. Cells for RT-PCR analysis were harvested and snap frozen in liquid nitrogen at 2, 7, 24, and 48 hours RNA was isolated by a silica-column based kit (RNEasy, Qiagen, Germany). RT-PCR was performed for IL-2, IL-15, CTLA4, GB, P, and FL as previously described.[1] Amplification was verified by addition of tissue-derived cDNA from rejecting kidneys known to express the target gene. Both proliferation and GAPDH expression served as indicators for the presence of viable cells.

RESULTS

IL-2, IL-7, and IL-15 were potent stimulators of PBMC proliferation and gene activation. Prestimulation with low-dose PHA in itself did not result in a marked proliferation, but enhanced the proliferative response to IL-2 (Fig 1) and IL-15. Without pretreatment, the proliferative response to IL-2 increased in a dose-dependent fashion but was less pronounced in the observed 72-hour period. T-cell growth factor transcripts were detectable at 2 and 7 hours and

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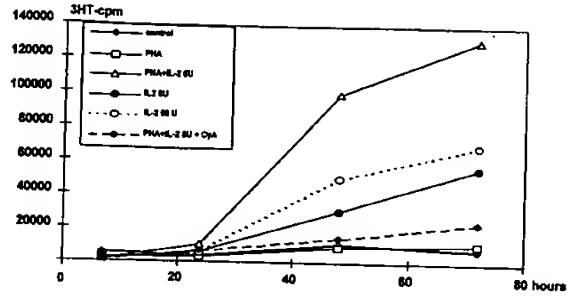

Fig. 1. Proliferation of PBMCs measured by 3H-thymidin incorporation. Prestimulation with 0.1 μg PHA/mL does not result in increased proliferation, but enhances the effect of IL-2 given 24 hours later. CyA almost completely blocks the proliferative response to IL-2, even in cells prestimulated with PHA.

peaked at 24 hours. CTL genes were inconsistently expressed at 7 hours and peaked at 24 hours.

CyA pretreatment caused a complete abrogation of IL-2-driven gene transcription for IL-2, IL-15, GB, P, and FL (Fig 2) and blocked the proliferative response (Fig 1). Similar effects were observed for Rapa and Dex, with the exemption of IL-2 transcription in presence of Rapa.

IL-15 driven proliferation and gene expression of IL-2, IL-7, IL-15, GB, P, and FL was not inhibited by CyA (Fig 2). In contrast, cells blocked by Dex or Rapa did not express transcripts for T-cell growth factors or CTL effectors after IL-15 stimulation. IL-15 induced IL-2 transcription in presence of Rapa.

IL-7 is not transcribed or expressed in PBMCs. Stimulation with IL-7 resulted in Rapa- and Dex-sensitive generation of IL-2 and CTL effectors. IL-15 gene transcription was inconsistent with positive expression in three of eight experiments. CyA did not inhibit IL-2 and CTL gene expression induced by IL-7 (Fig 2). IL-7 and IL-15 reciprocally induced transcription of other T-cell growth factors and stimulated generation of CTL effector transcripts in the presence of CyA.

CTLA4 gene transcription was not effectively prevented by any of the drugs tested.

DISCUSSION

Studies of intragraft gene activation in rejecting renal allografts have shown a specific and time-dependent pattern of immune activation gene transcription.[1–5] We and others[6] have failed to demonstrate IL-2 in rejecting grafts. Most of these investigations were performed in patients treated with CyA and Pred with or without azathioprine.

Pleiotropism and redundance are common features of the cytokine system, and there is accumulating evidence that in IL-2-depleted systems (CyA + Pred or IL-2 gene disruption) the antiallograft response is effectively supported by other T-cell stimulating factors, among which IL-4, IL-7, IL-9, IL-15, and IL-17 are possible candidates, although IL-9 (Li and Strom, unpublished) and IL-17[1] seem to be less likely. The IL-2, IL-7, and IL-15 signaling pathways have been shown to utilize both shared and unique components.[7,8]

Interference of T-cell activation by immunosuppressive drugs may cause a substantial change in the generation of immune mediators. Our experiments revealed a differential effect of CyA, Rapa, and Dex on the transcription of T-cell growth factors and cytotoxic effectors if added to PBMC cultures stimulated by T-cell growth factors. IL-15- and, less pronounced, IL-7-induced generation of CTL mediator transcripts was not blocked by CyA. CyA-resistent IL-7 gene activation has been previously reported in spleen cells,[9] but cannot be verified in purified PBMCs. Moreover,

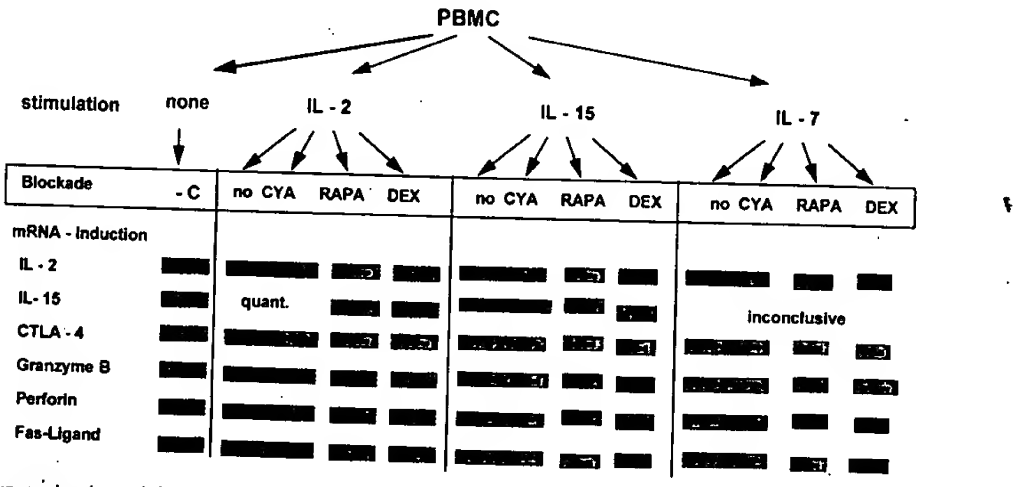

Fig. 2. Gene expression in peripheral blood cells stimulated with IL-2, IL-7, or IL-15 in the presence of CyA, Rapa, Dex, or no drug (postive control). Stimulation with any T-cell growth factor results in generation of T-cell transcripts, CTLA4, and CTL-effectors. None of the drugs inhibits CTLA-4 gene expression. The blocking effect of CyA seen in IL-2-driven proliferation cannot be seen in IL-15 or IL-7 stimulation. Rapa and Dex block granzyme B and perforin expression, but have less inhibitory effects on Fas ligand expression.

IL-7 induces development of a subpopulation of T cells that induces production of interferon (IFN)-gamma and IL-10 and potentiates the effects of CD28 costimulation on naive CD4 + cell proliferation.[10]

The data support previous observations that IL-15-driven T-cell proliferation[7] and gene and protein expression of the high-affinity IL-15a-receptor (10^{-11} pm) is abrogated by Dex and Rapa, but not by CyA.[11] All three known subforms of the alternativly spliced IL-15-Ra have been detected in CyA-treated kidney transplants.[12]

As recently shown, a similar pattern of IL-7 and IL-15 gene transcription was detected in rejected pancreatic islets in IL-2/IL-4 double knock-out mice,[13] and IL-15 mRNA has been detected in rejecting livers[14] and lungs[15] after transplantation. As CyA is not effective in blocking IL-7 and IL-15 gene expression, an effective regimen should combine/replace CyA with agents that block expression or action of these T-cell growth factors present in rejecting grafts.

A similar differential effect of drugs has been reported with TGF-b and IL-10, where both gene and protein expression can be induced by CyA,[16] whereas TGF-b seems to be blocked by tacrolimus via competition of FKBP12.[17]

Our knowledge of differential drug effects on T-cell activation is still fragmentary. We need more precise information concerning the pattern of gene activation in treatment of refractory, smoldering subacute, vascular, and chronic rejection. Moreover, the cellular environment of the transplanted organ may alter gene expression. Unlike in other organs, IL-5 has been described in rejecting livers.[16]

The knowledge of drug effects on the generation of relevant mediators in rejection will greatly facilitate choice of the appropriate regimen to effectively tailor maintenance and interventional immunosuppression to the patient's needs. An "antibiogram" of rejection needs definition of relevant gene expression in a given rejection episode (distinction between mediators and epiphenomena is essentials); and the effect of drugs on T-cell activation and mediator gene expression.

CONCLUSIONS

Knowledge of triggering forces in a given rejection episode and of the capacity of drugs to interfere with specific T-cell activating mechanisms may soon provide us with a tool to effectively tailor maintenance immunosuppression to the patient's needs.

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9. As in (*8*), we calculate the surface mass using the total incident velocity.
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Programmed Cell Death of T Cells Signaled by the T Cell Receptor and the α_3 Domain of Class I MHC

SURYAPRAKASH R. SAMBHARA AND RICHARD G. MILLER*

As well as being activated or rendered unresponsive, mature T lymphocytes can be deleted, depending on the signals received by the cell. Deletion by programmed cell death (apoptosis) is triggered if a T cell that has received a signal through its T cell receptor complex also receives a signal through the α_3 domain of its class I major histocompatibility complex (MHC) molecule. Such a signal can be delivered by a CD8 molecule, which recognizes the α_3 domain, or by an antibody to this domain. Precursors of both cytotoxic T lymphocytes (CTL's) and T helper cells are sensitive to this signal but become resistant at some point before completing differentiation into functioning CTL's or T helper cells. Because CTL's carry CD8, they can induce cell death in T cells that recognize them. This pathway may be important in both removal of autoreactive T cells and immunoregulation.

ACTIVATION OF A T LYMPHOCYTE requires occupancy of its antigen-specific cell surface receptor (TcR) by its appropriate ligand (processed antigen presented by class I or class II MHC molecules) and a second signal from a growth factor. If the second signal is not provided, the T cell becomes unresponsive (anergic) (*1*). We now present evidence for a pathway that leads to death of T lymphocytes that have been signaled through their TcR and then also receive a signal through the α_3 domain of their class I MHC molecule.

Short-term tissue culture studies of mouse lymphocytes in the mixed lymphocyte reaction (MLR) (*2*) have shown that CTL's can inactivate CTL precursors (CTLp's) that recognize them. This process does not involve the receptor of the CTL being recognized and can occur in the presence of cells and factors that can produce activation (*3*). This implies that, on being recognized, CTL's deliver a signal leading to inactivation; that is, they are acting as specialized antigen presenting cells (veto cells) (*4*) that inactivate T cells that recognize them. CTL's reactive to class I MHC (the majority) also carry the cell surface molecule CD8, which can recognize the α_3 domain of class I MHC (*5*). This binding can strengthen the adhesion of a CTL or CTLp to a cell it recognizes by binding to class I MHC molecules on that cell, thus facilitating T cell responses. We test here whether the CD8 molecule has a second function: Can a CD8 molecule on a cell being recognized by a T cell trigger the inactivation of that T cell by interacting with its class I MHC?

Paired $CD8^+$ and $CD8^-$ CTL lines of mouse origin were derived from two independent MLR with F_1(BALB/c × RNC) as responders and C57BL/6 (B6) as stimulators (*6*). All four lines had an αβ TcR, similar specificity (anti-D^b), and similar cytotoxic activity. The $CD8^+$ lines ($H\text{-}2^{d/k}$), when added to an MLR, reduced development of cytotoxic activity when BALB/c ($H\text{-}2^d$) were used as stimulators (MHC was shared with the added CTL's), but had no effect when SWR ($H\text{-}2^q$) were used as stimulators (no MHC sharing) (Fig. 1A). The $CD8^-$ CTL line had no effect on the response in either MLR.

We reasoned that covering the CD8 molecule on a $CD8^+$ CTL with a monoclonal antibody (MAb) to CD8 should block its ability to inactivate CTLp's. However, a MAb to CD8 would also block response induction (*7*). We therefore used a MAb specific for one of the two allelic forms of the mouse CD8 molecule (Ly-2.1 and Ly-2.2). MLR's were set up in which both responders (SJL and $H\text{-}2^s$) and stimulators (BALB/c and $H\text{-}2^d$) were Ly-2.2. To these were added CTL's from either BALB/c (identical to stimulator) or DBA/2 (same MHC but Ly-2.1) in the presence or absence of MAb to Ly-2.1. Both CTL lines produced equivalent response reduction in the absence of added MAb to Ly-2.1 but, as predicted, the response reduction produced by the $Ly\text{-}2.1^+$ DBA/2 CTL line was partly reversed by addition of the MAb to Ly-2.1 (Fig. 1B).

These results suggested that any cell line might inactivate CTLp's recognizing it if it were transfected with CD8. We compared the ability of a TcR^- $CD4^-$ $CD8^-$ BW 5147 T lymphoma line ($H\text{-}2^k$) to inactivate CTLp's reactive against $H\text{-}2^k$ with that of cells from the same line transfected with either CD8 (*8*) or CD4 (*9*). Appropriate expression of CD4, CD8, and $H\text{-}2^k$ was confirmed by flow cytometry (*10*). Only the $CD8^+$ lymphoma produced inhibition of the anti-$H\text{-}2^k$ MLR (Fig. 1C). This experiment provides direct evidence for the role of CD8 in the inactivation of CTLp's and also demonstrates that cells do not need to have the cytotoxic machinery of a CTL or to carry a TcR to be able to produce inactivation of CTLp's.

The MAb 34-2-12S (*11*), referred to as anti-α3, is known to bind to the α_3 domain of H-2 class I D^d MHC molecules (*12*). In that CD8 also binds to the α_3 domain, addition of this MAb to an MLR in which the responder cells carried D^d might mimic the effect of adding cells expressing CD8. The effects of adding anti-α_3 or control MAb on cell number, CTL activity, and interleukin-2 (IL-2) production in an MLR containing D^d-bearing responder cells were determined (experiment 1, Table 1). The control MAb's, of the same isotype (IgG2a) and generated in the same immunization protocol (*11*), all interact with class I MHC but appear not to interact with the α_3 domain (*12*). Anti-α3 prevented development of cytotoxic activity whereas the other MAb's to MHC had no effect (*13*). Surprisingly, both total cell number and IL-2 production [a measure of T helper cell (Th) activity] were also greatly reduced by anti-α3 but not by the other MAb's. One interpretation is that T helper precursors (Thp's), as well as CTLp's, are inactivated if they also receive a signal through the α_3 domain of their class I MHC molecule.

MAb 28-14-8S (*14*) is a second MAb known to bind to a class I MHC α_3 domain, in this case D^b (*15*). It also produced reduction of both cytotoxic activity and IL-2 production whereas control MAb's (*14, 15*) did not (experiment 2, Table 1A) (*16*).

The inactivation of CTLp's produced by added CTL's occurs early in an MLR; added CTL's have little or no effect if added later than 2 days after initiation (*3*). Similarly,

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anti-α3 completely inhibited cytotoxic activity in the MLR when added on days 0, 1, and 2, but had no effect when added on day 4 (Fig. 2A). The MAb completely inhibited IL-2 production when added on days 0 and 1 but had no effect when added on days 2 or 3 (Fig. 2B).

Access of anti-α_3 to the α_3 domain might be inhibited if MAb's to α_1/α_2 were first added. This, in fact, occurred when MAb 34-5-8S (anti-α_1/α_2) was added 48 hours after MLR initiation and anti-α_3 was added 1 hour later but not when they were added in reverse order (experiment 3, Table 1). This result provides evidence that the MAb to α_3 is not acting in a nonspecific manner and that it is acting through direct binding to MHC.

The TcR is associated with the CD3 molecular complex, thought to transmit signals from the TcR to the cytoplasm. An antibody to CD3 can act as a polyclonal T cell activator (*17*). Simultaneous addition of either MAb specific for α_3, 34-2-12S for H-2^d responders and 28-14-8S for H-2^b responders, but not other MAb's, to MHC produced inactivation as assessed by measurement of total cytotoxic activity (Table 1).

To ensure that anti-α3 spared T cells that had not received an activation signal, we developed a protocol in which, in a first incubation, only a fraction of all T cells would be inactivated by being given a signal through their TcR while being exposed to anti-α3. T cells were activated with the antibody KJ-16 [which recognizes the TcR-Vβ8.1 or −Vβ8.2 gene product present on about 20 percent of T cells (*18*)] in the presence or absence of anti-α3. When cultures were analyzed by flow cytometry, cells underwent blast transformation in response to KJ-16 (assessed by increase in the forward angle, light scatter signal) before undergoing cell death in response to anti-α_3 [assessed by uptake of the fluorescent DNA stain propidium iodide (*19*)]. By 40 hours after adding the KJ-16 alone, there was a significant increase in the total number of viable blasts compared to either control cultures with no added antibody or cultures that also contained anti-α_3 (Table 2). To test the ability of cultures treated with KJ-16 and anti-α_3 to respond to a different stimulus, T cells were again activated with KJ-16 in the presence or absence of anti-α_3. The antibodies were removed after 24 hours of incubation, and the cultures were exposed to MAb's to KJ-16 or CD3. Prior exposure to both KJ-16 and anti-α3 reduced the subsequent response to KJ-16 but had little effect on a subsequent response to MAb to CD3 (Table 2).

Signals that activate mature T cells can lead to programmed cell death of thymocytes (*20*). Death takes place by apoptosis, as indicated by the characteristic fragmentation of DNA. We compared DNA extracted from thymocytes and splenocytes. Exposure to MAb to CD3 plus anti-α3 but not MAb to CD3 alone appeared to induce fragmentation of splenocyte DNA comparable to that seen in DNA from thymocytes exposed

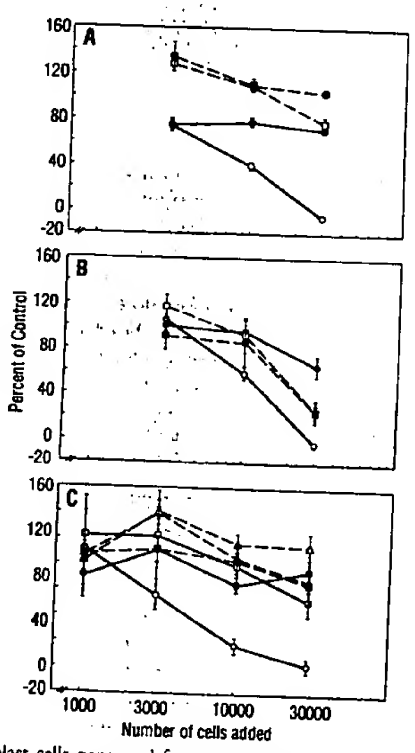

Fig. 1. Three experiments suggesting that CD8 mediates the inactivation of CTLp's by CTL's. (**A**) CD8$^+$, but not CD8$^-$, CTL's inactivated CTLp's. CD8$^+$ CD4$^-$ (○, □) or CD8$^-$ CD4$^-$ (●, ■) CTL's derived from an F_1(BALB/c × RNC) (H-2$^{d/k}$) anti-C57BL/6 (B6, H-2^b) MLR and added to SJL (H-2^s) anti-BALB/c (H-2^d) MLR's (solid line) or to SJL anti-SWR (H-2^q) MLR's (dashed line). (**B**) Covering CD8 molecules on a CD8$^+$ CTL line added to an MLR with a MAb to CD8 reduced the response reduction mediated by the CD8$^+$ CTL. The anti-B6-CTL DBA/2 (H-2^d, Ly-2.1) (○, ●) or the anti-B6 CTL BALB/c (H-2^d, Ly-2.2) (□, ■) were added to a MLR [SJL(Ly-2.2) anti-BALB/c (Ly-2.2)] in the absence (empty) or presence (filled) of antibody to Ly-2.1. (**C**) BW5147 lymphoma cells (H-2^k) transfected with the CD8α chain gene inhibited the generation of a CTL response in SJL anti-C3H (H-2^k) MLR. Untransfected BW cells (□, ■), BW-CD8 transfectants (○, ●), or BW-CD4 transfectants (△, ▲) were added to SJL anti-C3H MLR (empty) or SJL anti-DBA/2 (H-2^d) MLR (filled). MLR contained 1×10^5 responder lymph node cells and 3×10^5 irradiated (2000 rad) stimulator spleen cells in 200 μl of complete medium [α-minimal essential medium supplemented with fetal bovine serum (10%), 2-mercaptoethanol (10^{-5} M), and Hepes (10 mM) (*27*)]. CTL lines or BW transfectants were added in the numbers indicated on the abscissa. CTL activity was measured on day 5 in a 4-hour ^{51}Cr-release assay with ^{51}Cr-labeled target cells (1000 per culture) sharing MHC with the stimulator cells. Targets were either tumor cell lines or blast cells generated from normal spleen cells by incubation with concanavalin A (con A) at 2 μg/ml for 48 hours (*27*). Specific lysis was calculated and re-expressed as "cytotoxic activity" which is proportional to the number of CTL's present (*32*). A value of 0.1 corresponds to 10% specific lysis; 1.0 to 63% specific lysis. Each value represents the mean ± SD of five replicates and is expressed relative to a control to which CTL lines or BW transfectants were not added. Antibody to Ly-2.1 (Cedarlane Laboratories) was used at 1/40 dilution, the optimal dilution to inhibit an MLR response against C57BL/6 stimulator cells by DBA/2 but not BALB/c responder cells.

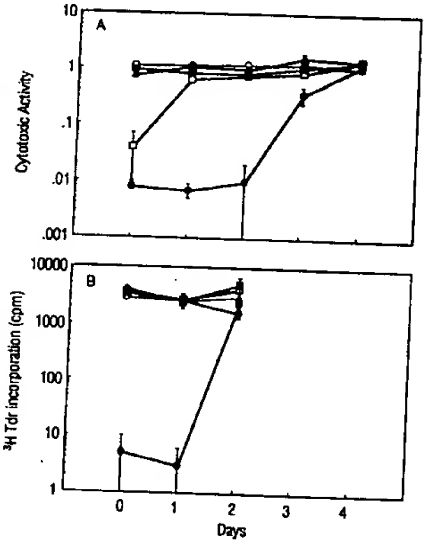

Fig. 2. Inhibition of CTL (**A**) and Th (**B**) responses in an MLR response of BALB/c against B6 by addition of MAb against the α_3 domain of class I MHC added at various times after culture initiation as indicated. Cultures were prepared and assayed as in Fig. 1. Antibodies added were as follows: none (○), anti-α_3 of H-2D^d (34-2-12S, ●), anti α_1 or -α_2 of H-2D (34-5-8S, □), anti H-2D^d (34-4-20S, ▲), or anti H-2K^{d}D^d (34-1-2S, ■) at concentrations indicated in Table 1.

Fig. 3. The anti-α3 signal leads to cell death by apoptosis. DBA/2 thymocytes or spleen cells (10^7 per culture) were cultured in 10 ml of complete medium (see legend to Fig. 1) for 24 hours with antibody to CD3 (145-2C11) or that antibody and anti-α3 (34-2-12S). Viable cells were harvested and total cellular DNA was extracted with phenol-chloroform (*33*). The DNA (3 μg per lane) was subjected to electrophoresis for 7 hours at 30 V in 2% agarose gel with tris-borate buffer (*34*) at 37°C and was stained with ethidium bromide. Left lane, thymocytes cultured with anti-CD3; middle lane, spleen cells cultured with anti-CD3; right lane, spleen cells cultured with anti-CD3 plus anti-α_3.

Table 1. Inhibition of T cell generation by a monoclonal antibody to α_3*. Monoclonal antibodies to MHC were added as indicated to either MLR prepared as in Fig. 1 or spleen cells (10^6 per well) cultured in 200 μl of complete medium with anti-CD3 [145-2C11 (*30*), 2.5 μg/ml]. The MLR cultures were assayed for IL-2 content with a CTLL assay and for cytotoxic activity as described (*27*). The anti-CD3 activated cultures were assayed for cytotoxic activity with ^{51}Cr-labeled EL-4 thymoma cells (10^3) and con A (1 μg/ml); the con A was used to overcome the need for specific recognition in the CTL-target cell interaction and enables the detection of CTL's of all specificities (*31*). All MAb's were from hybridoma culture supernatants. They were included at final concentrations of 1 to 3 μg/ml, which gave equivalent cell surface staining of lymphocytes by fluorescence analysis. All entries are mean ± SD of five replicates. Each experiment was repeated at least three times with the same result.

Antibody added	Determinant recognized	MLR cultures			Anti-CD3 activated cultures cytotoxic activity	
		Cell recovery on day 3 (% of control)	IL-2 on day 3 (units/ml)	Cytotoxic activity on day 5	48 hours*	72 hours*
		Expt. 1: BALB/c (H-2^d) responding to B6 (H-2^b)			*Expt. 1: DBA/2 spleen cells*	
None		100	0.79 ± 0.19	1.19 ± 0.11	1.80 ± 0.23	1.12 ± 0.06
34-2-12S (anti-α_3)	α_3 of D^d	30 ± 10	<0.01	−0.01 ± 0.01	0.30 ± 0.03	−0.01 ± 0.01
34-5-8S	α_1 or α_2 of D^d	96 ± 9	1.33 ± 0.50	1.01 ± 0.11	1.72 ± 0.23	0.97 ± 0.07
34-4-20S	D^d	117 ± 4	1.45 ± 0.60	0.79 ± 0.08	1.63 ± 0.26	ND
34-1-2S	K^dD^d	108 ± 4	1.70 ± 0.38	0.79 ± 0.10	1.66 ± 0.42	0.92 ± 0.10
		Expt. 2: B6 (H-2^b) responding to DBA/2 (H-2^d)			*Expt. 2: B6 spleen cells*	
None		100	1.75 ± 0.65	1.43 ± 0.17		1.90 ± 0.25
28-14-8S	α_3 of D^b	50 ± 6	<0.10	−0.03 ± 0.03		−0.04 ± 0.01
20-8-4S	α_1 of K^b	118 ± 11	1.70 ± 0.56	1.89 ± 0.44		ND
28-8-6S	K^bD^b	111 ± 9	1.03 ± 0.09	1.47 ± 0.24		0.93 ± 0.05
		Expt. 3: DBA/2 (H-2^d) responding to B6 (H-2^b)				
None				0.76 ± 0.14		
34-2-12S	α_3 of D^d			0.07 ± 0.08		
34-2-12S plus 34-5-8S (1 hour before)†	α_1 or α_2 of D^d + α_3 of D^d			0.68 ± 0.10		
34-2-12S plus 34-5-8S (1 hour after)†	α_3 of D^d + α_1 or α_2 of D^d			0.03 ± 0.02		

*Independent experiments. †One MAb was added after 48 hours of culture, the second 1 hour later.

to MAb to CD3 alone (Fig. 3).

We conclude that either CD8 or anti-α3 can induce death in either a CTLp or Thp at some point after it has been signaled through its TcR-CD3 complex but before it has become mature (*21*). This appears to be a result of a specific signal induced through the α_3 domain of the class I MHC molecule. Our data are consistent with results with a human $CD8^+$ CTL line and a stable $CD8^-$ antisense transfectant of this line. The $CD8^+$, but not the $CD8^-$ line, could down-regulate proliferative and cytotoxic responses against its own MHC antigens (*22*). A tumor cell line capable of stimulating an MLR down-regulates both $CD4^+$ and $CD8^+$ cells capable of recognizing it if the line is transfected with CD8 (*23*). In $CD4^+$ T cells (*24*), death is induced if CD4 molecules are cross-linked and the cell is then signaled through its TcR.

Programmed cell death may provide a mechanism for deleting self-reactive cells, provided that potentially immunogenic peptides are recognized in association with MHC on $CD8^+$ cells before being recognized on antigen presenting cells that allow activation. CD8-mediated deletion may also play a role in down-regulation of an immune response by deletion of activated Thp. This would require that the $CD8^+$ cell also carry class II MHC so that the Thp could recognize it. Resting T cells do not express class II MHC but at least some activated human T cells can be induced to express class II MHC (*25*) and the same may be true for mouse (*26*). Class II MHC–reactive Thp can be inactivated in vivo within 3 days of injection of allogenic lymphoid cells (*27*).

It is well established that there are $CD8^-$ cells, such as NK cells (*28*) and a $Thy\text{-}1^+$ cell subset in bone marrow (*29*) that inactivate CTLp's that recognize them. If these cells act by signaling through the α_3 domain of class I MHC, they must carry some other

Table 2. Deletion of activated T cells by monoclonal antibody to α_3. DBA/2 spleen cells (10^7 per well for cell recovery or 10^6 per well for proliferation) were cultured as in Table 1 (for anti-CD3, activation cultures), and antibodies (as indicated) were included at the start of culture. To assess cell recovery, viable cells (total and blast) were counted after 40 hours. To monitor proliferation, after 24 hours cultures either were washed three times with complete medium, incubated 16 hours, restimulated with either KJ-16 or 145-2C11 and incubated another 24 hours, the last 18 hours with [^{3}H]thymidine, or were left undisturbed except for a final 18-hour incubation with [^{3}H]thymidine (control).

Antibody added initially	Cell recovery* [viable cells (10^4 cells)]		Proliferation [radioactivity incorporated (cpm)]†		
	Total	Blast	+KJ-16 (anti-Vβ8.1 - 8.2)	+145-2C11 (anti-CD3)	Control
None	256 ± 22	27 ± 2	5,179 ± 667	23,147 ± 1,059	3,823 ± 572
KJ-16	768 ± 39	210 ± 11	6,979 ± 368	25,451 ± 2,551	8,114 ± 510
KJ-16 + 34-2-12S (anti-α_3)	350 ± 25	65 ± 5	2,524 ± 264	24,442 ± 1,370	1,331 ± 168
KJ-16 + 34-5-8S (anti-α_1 or -α_2)	ND	ND	6,099 ± 715	23,517 ± 788	6,018 ± 677

*Entries are based on replicate visual counts and percentages based on flow cytometric analysis of 20,000 cells. †Entries are mean (cpm) ± SD of five replicate cultures.

cell surface molecule that can recognize this domain.

The observation that only those CTLp's and Thp's that have been signaled through their antigen-specific surface receptors are killed by exposure to an antibody recognizing all lymphocytes (anti-α3) may prove useful for establishing tolerance in the adult animal to a particular antigen.

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CREB: A Ca^{2+}-Regulated Transcription Factor Phosphorylated by Calmodulin-Dependent Kinases

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The mechanism by which Ca^{2+} mediates gene induction in response to membrane depolarization was investigated. The adenosine 3′,5′-monophosphate (cAMP) response element–binding protein (CREB) was shown to function as a Ca^{2+}-regulated transcription factor and as a substrate for depolarization-activated Ca^{2+}-calmodulin–dependent protein kinases (CaM kinases) I and II. CREB residue Ser^{133} was the major site of phosphorylation by the CaM kinases in vitro and of phosphorylation after membrane depolarization in vivo. Mutation of Ser^{133} impaired the ability of CREB to respond to Ca^{2+}. These results suggest that CaM kinases may transduce electrical signals to the nucleus and that CREB functions to integrate Ca^{2+} and cAMP signals.

DNA ELEMENTS THAT CONTAIN THE consensus sequence TGACGTCA were originally identified as cAMP response elements (CREs) in neuropeptide genes (*1*), but recent studies in neuronal cell lines have mapped inducibility by membrane depolarization and increased intracellular Ca^{2+} concentrations to similar sequences in the c-*fos* (*2–4*) and proenkephalin genes (*5*). Depolarization of PC12 pheochromocytoma cells is correlated with rapid phosphorylation of the CRE-binding protein CREB on amino acid residue Ser^{133} (*4*). Phosphorylation of Ser^{133} stimulates the ability of CREB to activate gene transcription (*6*), suggesting that CREB may mediate transcriptional induction by Ca^{2+} as well as by cAMP. However, because multiple CRE-binding proteins exist (*7, 8*), distinct members of this family may separately confer Ca^{2+} and cAMP inducibility on the CRE (*4*). To determine whether CREB functions as a Ca^{2+}-activated transcription factor and to test if phosphorylation of Ser^{133} is important for this activation, we have targeted CREB to a different DNA regulatory sequence by fusing it to the DNA-binding and dimerization domain of the yeast transcriptional activator GAL4 (*9*) (Fig. 1). The use of a reporter gene that contains GAL4 binding sites allowed a spe-

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The Role of the Common Cytokine Receptor γ-Chain in Regulating IL-2-Dependent, Activation-Induced CD8$^+$ T Cell Death[1]

Zhenhua Dai, Alexandr Arakelov, Maylene Wagener, Bogumila T. Konieczny, and Fadi G. Lakkis[2]

IL-2-dependent, activation-induced T cell death (AICD) plays an important role in peripheral tolerance. Using CD8$^+$ TCR-transgenic lymphocytes (2C), we investigated the mechanisms by which IL-2 prepares CD8$^+$ T cells for AICD. We found that both Fas and TNFR death pathways mediate the AICD of 2C cells. Neutralizing IL-2, IL-2Rα, or IL-2Rβ inhibited AICD. In contrast, blocking the common cytokine receptor γ-chain (γc) prevented Bcl-2 induction and augmented AICD. IL-2 up-regulated Fas ligand (FasL) and down-regulated γc expression on activated 2C cells in vitro and in vivo. Adult IL-2 gene-knockout mice displayed exaggerated γc expression on their CD8$^+$, but not on their CD4$^+$, T cells. IL-4, IL-7, and IL-15, which do not promote AICD, did not influence FasL or γc expression. These data provide evidence that IL-2 prepares CD8$^+$ T lymphocytes for AICD by at least two mechanisms: 1) by up-regulating a pro-apoptotic molecule, FasL, and 2) by down-regulating a survival molecule, γc. *The Journal of Immunology*, 1999, 163: 3131–3137.

Interleukin-2 promotes the proliferation of T lymphocytes following primary activation with Ag by binding to a high affinity receptor, IL-2R, which consists of three subunits: IL-2Rα, IL-2Rβ, and the common cytokine receptor γ-chain (γc)[3] (1). Paradoxically, IL-2 also programs T lymphocytes for activation-induced cell death (AICD) (2) after repeated antigenic stimulation (2–7). The AICD of mature CD4$^+$ T lymphocytes is mediated by Fas-Fas ligand (FasL) interactions (8–11). IL-2 prepares these cells for AICD by up-regulating FasL expression and down-regulating the transcription of FLIP (IL-1β-converting enzyme-like protease-like inhibitory protein), a protein that inhibits Fas-mediated apoptosis (12). The AICD of CD8$^+$ T lymphocytes is mediated mainly by the TNFR cell death pathway (13–15), but Fas plays a critical role in the AICD of autoreactive CD8$^+$ T cells (16). The cellular mechanisms by which IL-2 prepares CD8$^+$ T lymphocytes for AICD are not known. These mechanisms may differ from those in the CD4$^+$ population, because efficient elimination of effector CD8$^+$ T cells is necessary to avoid nonspecific injury of tissues in which the inciting Ag, for example a viral protein, persists.

γc is crucial for CD8$^+$ T cell survival. It is expressed on naive and activated CD4$^+$ and CD8$^+$ T cells; is a shared subunit of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors; and is central to cytokine-mediated T cell proliferation (17, 18). γc mutations in humans result in severe combined immunodeficiency characterized by a profound decrease in circulating T lymphocytes (19). γc gene-knockout mice exhibit severely defective lymphoid development (20–22). At birth, NK cells are absent, and mature B and T lymphocytes are markedly diminished, indicating that γc is indispensable for the development of all murine lymphocyte classes. Although activated CD4$^+$ T cells accumulate over time in γc-deficient mice, the CD8$^+$ population remains extremely small (20, 23). These observations suggest that mature CD4$^+$ T cells respond to γc-independent mitogens, whereas mature CD8$^+$ T cells are critically dependent on γc for proliferation and survival. The finding that female γc-deficient mice made transgenic (tg) for a TCR specific for the HY male Ag lack mature CD8$^+$ TCR-tg T lymphocytes in their peripheral lymphoid organs despite efficient positive selection of these cells in the thymus further indicates that γc provides essential survival signals to CD8$^+$ T cells (24).

The presence of multiple surface proteins that influence CD8$^+$ T lymphocyte survival raises the possibility that IL-2 could prepare these cells for AICD by up-regulating death-promoting molecules such as FasL and down-regulating survival-promoting molecules such as γc. To test this hypothesis, we established an Ag-specific, IL-2-dependent AICD model using CD8$^+$ TCR-tg T cells (2C), which recognize the L^d murine MHC class I Ag (25). In this model, 2C cells primed in vivo with L^d-expressing splenocytes underwent apoptosis upon cross-linking of their tg TCR in vitro with a clonotypic Ab (1B2). AICD occurred only if IL-2 was present in the medium. Using this model, we provide evidence that IL-2 sensitizes CD8$^+$ T cells to AICD by at least two feedback mechanisms: 1) by up-regulating FasL expression, and 2) by down-regulating γc expression on activated CD8$^+$ T lymphocytes.

Materials and Methods

Mice

Wild-type and IL-2 gene knockout (IL-2$^{-/-}$) (26) C57BL6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). 2C TCR-tg mice (C57BL6 background) (27) were provided by Dr. Dennis Loh (Washington

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[3] Abbreviations used in this paper: γc, common cytokine receptor γ-chain; AICD, activation-induced cell death; FasL, Fas ligand; IL-2$^{+/+}$, wild-type mice; IL-2$^{-/-}$, IL-2 gene-knockout mice; tg, transgenic; FLIP, IL-1β-converting enzyme-like protease-like inhibitory protein.

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University, St. Louis, MO) and were bred at the Veterans Administration Medical Center/Emory University animal facility. Screening for the 2C transgene was performed by PCR.

Cytokines and Abs

Endotoxin-free recombinant mouse IL-2, mouse IL-4 (3.4×10^7 U/mg), mouse IL-7 (5×10^6 U/mg), and simian IL-15 (2.2×10^8 U/mg) were purchased from Genzyme (Cambridge, MA). Monoclonal rat anti-mouse IL-2, rat anti-mouse IL-4, and hamster anti-mouse TNF-α were also purchased from Genzyme. Monoclonal rat anti-mouse γc (3E12 and 4G3) (28), rat anti-mouse IL-2Rα (3C7), rat anti-mouse IL-2Rβ (TM-β1), isotype control rat IgG2a and rat IgG2b, and hamster anti-mouse FasL (MFL3) were purchased from PharMingen (San Diego, CA). The mouse hybridoma cell line producing the clonotypic Ab 1B2 that is specific for the 2C tg TCR was provided by Dr. Dennis Loh (Washington University) (27). Hybridoma supernatant or isotype control mIgG1 (PharMingen) were used to coat culture wells.

Cell preparation

To study alloantigen-specific responses of $CD8^+$ TCR-tg T lymphocytes, 2C C57BL/6 ($H\text{-}2^b$) mice were either left naive or were primed in the footpads and i.p. with 1×10^7 BALB/c ($H\text{-}2^d$) splenocytes/injection. Five days later, the mice were sacrificed, and the lymph node and spleen cells were isolated, pooled, and enriched for T lymphocytes by nonadherence to nylon wool columns (Polysciences, Warrington, PA). $CD4^+$ T cells, B cells, monocytes, granulocytes, and NK cells were then eliminated by incubation with monoclonal rat anti-mouse CD4 (GK1.5), mouse anti-mouse CD59 (clone 2B4; PharMingen), and rat anti-mouse HSA (J11D; PharMingen), followed by addition of guinea pig complement (Accurate Chemical & Scientific, Westbury, NY). The remaining cell population was >95% $CD8^+$, of which >98% expressed the 2C tg TCR ($1B2^+$) as determined by flow cytometry. The enriched $CD8^+1B2^+$ cells, referred to as 2C cells in this manuscript, were then used in the AICD assay. The $CD8^+$ T cell enrichment protocol was also applied to wild-type (nontransgenic) and $IL\text{-}2^{-/-}$ C57BL/6 mice when indicated.

AICD assay

$CD8^+1B2^+$ T lymphocytes (1×10^6) isolated from primed 2C mice as described in the previous section were cultured in 24-well plates precoated with hamster anti-mouse CD28 (37.51; PharMingen) and 1B2 hybridoma supernatant. Control wells were precoated with isotype control mouse IgG1 (PharMingen) instead of 1B2. Complete RPMI 1640 medium (10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) was used. Cytokines, cytokine-neutralizing Abs, or anti-cytokine receptor Abs were added at the beginning of the culture as indicated. Twenty-four hours later, cells were washed, counted, and analyzed for apoptosis or for cell surface markers by flow cytometry as described below. To study AICD in vivo, 2C mice were primed in the footpads with 1×10^7 BALB/c ($H\text{-}2^d$) splenocytes on days 0 and 5. Twenty-four hours later, popliteal and inguinal lymph node cells were pooled, enriched for $CD8^+1B2^+$ cells as described in the previous section, and analyzed for apoptosis or for cell surface markers by flow cytometry.

Flow cytometry

To detect apoptosis, cells were fixed in 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, and labeled with fluorescein-tagged dUTP by the TUNEL method according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Boehringer Mannheim, Mannheim, Germany). Cells were then analyzed by single-color flow cytometry on a Becton Dickinson FACScan (Mountain View, CA). The total lymphocyte population was gated, and apoptosis was measured by calculating the percentage of $TUNEL^+$ cells. To determine the absolute number of apoptotic cells, the percentage of $TUNEL^+$ cells was multiplied by the total number of cells present in each well at the end of the experiment. To measure cell surface markers, cells were stained with PE- or FITC-conjugated rat anti-CD4 (H129.19; PharMingen), PE- or FITC-conjugated rat anti-mouse CD8 (53-6.7; PharMingen), FITC-conjugated rat anti-mouse IL-2Rβ (TM-β1; PharMingen), PE-conjugated rat anti-mouse γc (4G3; PharMingen), or PE-conjugated hamster anti-mouse FasL (MFL3; PharMingen). To detect intracellular Bcl-2 expression, cells were fixed in 1% paraformaldehyde and permeabilized with 0.1% Triton X-100 before staining with FITC-conjugated hamster anti-mouse Bcl-2 (3F11; PharMingen). The appropriate conjugated, isotype control Abs were used as negative controls. Stained cells were analyzed by single- or dual-color flow cytometry on a Becton Dickinson FACScan.

Proliferation assay

$CD8^+1B2^+$ T lymphocytes isolated from primed 2C mice were cultured in triplicate at 37°C in a 5% CO_2 incubator in complete RPMI medium (10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) in 96-well plates (2.5×10^5 cells/well) precoated with 1B2 hybridoma supernatant and hamster anti-mouse CD28 (37.51; PharMingen). mAbs to IL-2 and the IL-2R subunits were added at the beginning of the culture as indicated. Twenty-four hours later, the wells were pulsed with 0.5 μCi of [^{3}H]TdR and harvested after 6 h onto fiberglass filter papers (PhD cell harvester; Cambridge Technology, Cambridge, MA). [^{3}H]TdR uptake was measured in a scintillation counter (Beckman, Palo Alto, CA).

Results

IL-2-dependent AICD of $CD8^+$ T cells

We established an AICD model driven by repeated stimulation of TCR-tg mature $CD8^+$ T cells (2C). 2C cells react specifically to the L^d MHC class I Ag, and their TCR is recognized by a clonotypic Ab (1B2) (25). Splenocytes of either naive 2C mice or 2C mice primed with L^d-expressing splenocytes were enriched for $CD8^+1B2^+$ T cells and cultured in complete medium in the presence of either plate-bound 1B2 Ab or isotype control mIgG. Apoptosis was measured by flow cytometry 24 h later. As shown in Fig. 1*A*, a small proportion of naive 2C cells underwent apoptosis when their TCR were cross-linked in vitro with 1B2. This degree of apoptosis was most likely due to passive cell death, because a similar proportion of naive 2C cells underwent apoptosis when challenged with mIgG instead of 1B2 (Fig. 1*A*). In contrast, in vitro stimulation with 1B2 increased the percentage of apoptotic cells when 2C cells were preactivated in vivo by challenging 2C mice with L^d-expressing splenocytes (Fig. 1*A*). Unlike 1B2, mIgG did not increase the apoptosis of preactivated 2C cells, indicating that AICD in this model is dependent on repeated stimulation of T lymphocytes via their TCR.

We then tested the effect of T cell mitogenic cytokines on the AICD of 2C cells. Recombinant cytokines were added to preactivated 2C cells at the time of in vitro stimulation with either plate-bound 1B2 or mIgG. IL-2 increased the 1B2-induced apoptosis of preactivated 2C cells significantly, whereas the addition of IL-4, IL-7, and IL-15 reduced the percentage of apoptotic cells (Fig. 1*B*). Excess IL-2-neutralizing Ab, but not control rat IgG or IL-4-neutralizing Ab, completely inhibited 1B2-induced apoptosis of preactivated 2C cells (Fig. 1*C*). The data indicate that the AICD of $CD8^+$ T cells is dependent on IL-2.

To determine whether IL-4, IL-7, and IL-15 reduce the proportion of apoptotic cells by preventing AICD or by increasing T cell proliferation, we calculated the absolute number of apoptotic 2C cells following the induction of AICD (Fig. 1*D*). We found that IL-2 increases the number of apoptotic cells significantly, whereas IL-4 and IL-7 reduced the number of apoptotic cells by a small, but significant, amount (19–24% reduction). IL-15 did not have a significant effect on AICD. These data suggest that IL-4 and IL-7 have a modest antiapoptotic effect on activated $CD8^+$ T cells.

Counting apoptotic cells is subject to error, because dead cells can degenerate in culture. We therefore quantitated the absolute number of viable cells 24 h after in vitro stimulation of $CD8^+$ T lymphocytes obtained from either naive or primed 2C mice (Fig. 1*E*). The number of viable 2C lymphocytes obtained from naive 2C mice increased significantly following their stimulation with 1B2 alone or with 1B2 plus IL-2, IL-4, IL-7, or IL-15. Addition of anti-IL-2 inhibited 1B2-induced expansion of these cells. In contrast, the number of viable 2C lymphocytes obtained from primed 2C mice did not increase significantly when stimulated with 1B2 alone or with 1B2 plus IL-2. The addition of IL-4, IL-7, or IL-15 restored 1B2-induced expansion of these cells. These data are also

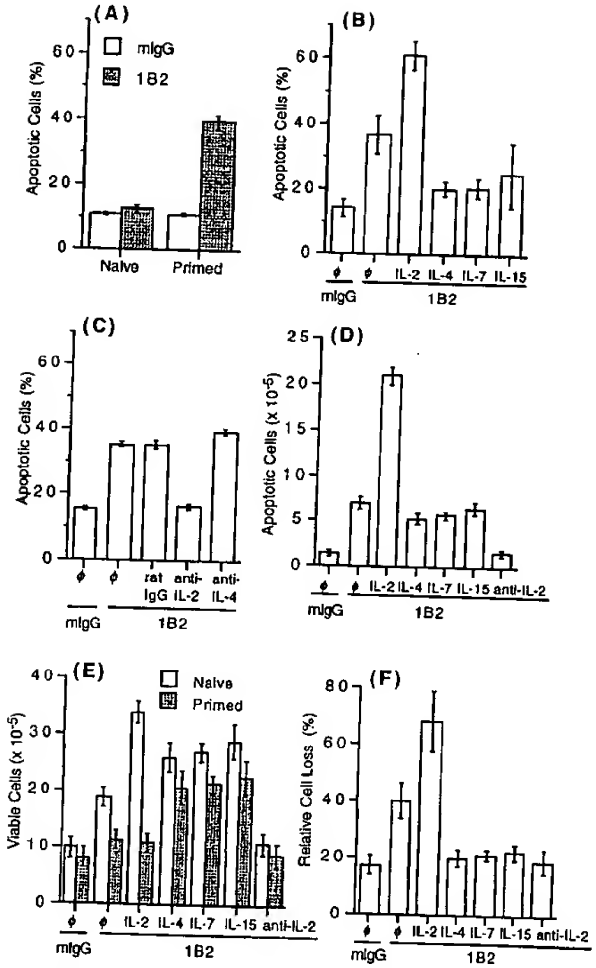

FIGURE 1. IL-2-dependent AICD of $CD8^+$ T cells. *A*, T cell activation is required for AICD. 2C ($CD8^+1B2^+$) cells, isolated from either naive 2C mice or 2C mice primed with L^d-bearing splenocytes, were stimulated in vitro with either TCR-cross-linking Ab (1B2) or isotype control Ab (mIgG). Twenty-four hours later, cells were labeled by the TUNEL method and analyzed by single-color flow cytometry. After gating on the total lymphocyte population, the proportion (percentage) of apoptotic ($TUNEL^+$) cells was calculated. The mean ± SD of three experiments are shown. *B*, Exogenous IL-2 enhances AICD. 2C cells were isolated from primed 2C mice, stimulated in vitro with either mIgG (control) or 1B2, and analyzed for apoptosis as described in *A*. IL-2 (50 U/ml), IL-4 (20 ng/ml), IL-7 (20 ng/ml), or IL-15 (20 ng/ml) was added at the beginning of the culture. The mean ± SD of four experiments are shown. ϕ, no cytokine added. *C*, Endogenous IL-2 is required for AICD. 2C cells were isolated from primed 2C mice, stimulated in vitro with either mIgG (control) or 1B2, and analyzed for apoptosis as described in *A*. Isotype control (rat IgG) or cytokine-neutralizing Abs (10 μg/ml each) were added at the beginning of the culture. The mean ± SD of three experiments are shown. *D*, Effect of T cell mitogenic cytokines on AICD. The AICD of 2C cells was studied as described in *B* and *C*. The absolute number of apoptotic 2C cells was calculated by multiplying the percentage of $TUNEL^+$ cells by the total number of cells present in each well at the end of the experiment. The mean ± SD of three experiments are shown. *E*, Effect of T cell mitogenic cytokines on the number of viable naive (open bars) or primed (solid bars) 2C cells 24 h following in vitro stimulation with mIgG or 1B2. Cell viability was determined by trypan blue exclusion. The mean ± SD of three experiments are shown. *F*, Effect of T cell mitogenic cytokines on the relative loss of primed 2C cells following in vitro stimulation with mIgG or 1B2. Relative cell loss (percentage) in each experimental condition was calculated based on cell numbers shown in *E* according to the following formula: (open bar − solid bar/open bar) × 100.

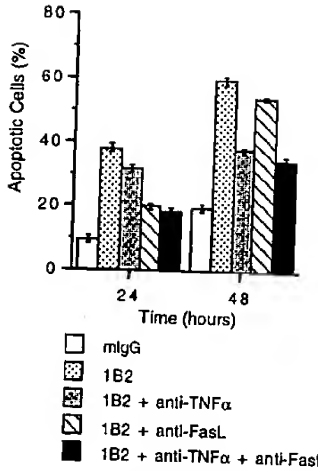

FIGURE 2. Blocking FasL or TNF-α inhibits IL-2-dependent AICD of $CD8^+$ T cells. 2C cells were isolated from primed 2C mice and stimulated in vitro with either mIgG (control) or 1B2. Anti-TNF-α (10 μg/ml), anti-FasL (10 μg/ml), or both Abs were added at the beginning of the culture. Twenty-four and forty-eight hours later, cells were labeled by the TUNEL method and analyzed by single-color flow cytometry. After gating on the total lymphocyte population, the proportion (percentage) of apoptotic (TUNEL+) cells was calculated. The mean ± SD of three experiments are shown.

portrayed as relative cell loss (percentage), which reflects the difference in cell expansion between 2C lymphocytes obtained from primed mice and those obtained from naive mice (Fig. 1*F*). These findings confirm the unique pro-apoptotic effect of IL-2 on activated $CD8^+$ T cells.

Blocking FasL or TNF-α inhibits IL-2-dependent AICD of $CD8^+$ T cells

To test whether the IL-2-dependent AICD of $CD8^+$ T lymphocytes is mediated by the Fas and/or the TNFR death pathways, we studied the effect of FasL- and TNF-α-blocking Abs on AICD in our model. In vivo activated 2C cells were stimulated in vitro with plate-bound 1B2 Ab, and the percentage of apoptotic cells was determined 24 and 48 h later. We found that the addition of excess TNF-α-neutralizing Ab reduced AICD at 24 h by about 20%, whereas blocking FasL-Fas interactions with anti-FasL Ab inhibited AICD by about 60% (Fig. 2). In contrast, TNF-α neutralization resulted in more inhibition of AICD at 48 h than that achieved by blocking FasL (~55 vs ~10%). Combined blockade of FasL and TNF-α did not result in additive or synergistic inhibition of AICD at 24 or 48 h, suggesting that the roles of Fas and TNFR in $CD8^+$ T cell apoptosis are separated temporally.

IL-2 up-regulates FasL expression on activated $CD8^+$ T cells

IL-2 up-regulates TNFR expression on $CD4^+$ and $CD8^+$ T cells (29, 30), suggesting that IL-2 sensitizes activated T cells to apoptosis via the TNFR death pathway. IL-2 up-regulates the surface expression of FasL on $CD4^+$ T cells (12), but its effect on FasL expression on $CD8^+$ T cells is not known. To address this issue, 2C mice were primed with L^d-bearing splenocytes. Five days later, their spleen cells were enriched for $CD8^+1B2^+$ T cells and restimulated in vitro with plate-bound 1B2 Ab or isotype control mIgG. FasL expression was measured 24 h later by flow cytometry. As shown in Fig. 3, activation of 2C cells with plate-bound 1B2 up-regulated FasL expression. Adding rIL-2 enhanced FasL expression further, whereas IL-2-neutralizing Ab inhibited FasL up-regulation (Fig. 3*A*). In contrast to IL-2, cytokines that do not

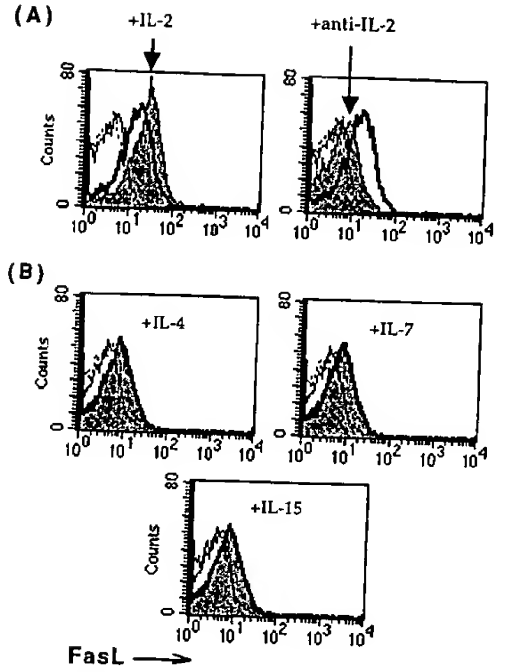

FIGURE 3. IL-2 up-regulates FasL expression on activated CD8$^+$ T cells. In both experiments (*A* and *B*), 2C cells were isolated from primed 2C mice and stimulated in vitro with either mIgG (control) or 1B2. IL-2 (50 U/ml), anti-IL-2 (10 μg/ml), IL-4 (20 ng/ml), IL-7 (20 ng/ml), or IL-15 (20 ng/ml) was added at the beginning of the culture. Twenty-four hours later, surface expression of FasL was measured by single-color flow cytometry: unstained cells (dotted line), mIgG-stimulated cells (solid line), 1B2-stimulated cells (bold line), and 1B2-stimulated cells treated with cytokines or IL-2-neutralizing Ab (shaded histogram).

promote AICD (IL-4, IL-7, and IL-15) did not alter FasL expression on activated 2C cells (Fig. 3*B*).

γc-blocking Abs enhance IL-2-dependent AICD of CD8$^+$ T cells and inhibit Bcl-2 induction

To test whether γc regulates IL-2-dependent AICD of CD8$^+$ T cells, we examined the effect of γc-blocking Abs on lymphocyte apoptosis in our model. Abs that target γc (3E12 and 4G3), but do not interfere with the binding of IL-2 to the α and β subunits of the IL-2R (28), enhanced AICD in the 2C cell population (Fig. 4*A*). Enhanced AICD was confirmed by determining the absolute number of apoptotic cells in this experiment. A 34% increase in the absolute number of apoptotic cells was observed following the addition of γc-blocking Abs to 1B2-stimulated 2C cells (mean of three experiments). Dose-dependent augmentation of AICD was observed when the concentrations of 3E12 and 4G3 were varied between 0.1–100 μg/ml (data not shown). If rIL-2 was added to the medium along with 3E12 and 4G3, the proportion of apoptotic cells increased further, confirming that IL-2 interacts with its receptor and promotes AICD in the presence of γc-blocking Abs (Fig. 4*A*). In contrast, Abs that block the α and β subunits of the IL-2R inhibited AICD completely (Fig. 4*A*). Enhanced apoptosis induced by γc-blocking Abs was not due to increased passive cell death, because these Abs did not alter the apoptosis of naive 2C cells cultured in the presence of plate-bound mIgG (25.3, 25.8, 26.0, and 25.6% apoptosis in the presence of rat IgG isotype control, anti-IL-2Rα, anti-IL-2Rβ, and anti-IL-2Rγ, respectively) or plate-bound 1B2 (26.2, 27.2, 26.1, and 29.8% apoptosis in the presence of rat IgG isotype control, anti-IL-2Rα, anti-IL-2Rβ, and anti-IL-2Rγ, respectively). Although anti-IL-2Rα, anti-IL-2Rβ, and anti-γc Abs inhibited 1B2-induced proliferation of preactivated 2C cells (Fig. 4*B*), only anti-γc Abs enhanced their apoptosis (Fig. 4*A*). These observations indicate that γc is essential for both the proliferation and the survival of activated CD8$^+$ T cells. To

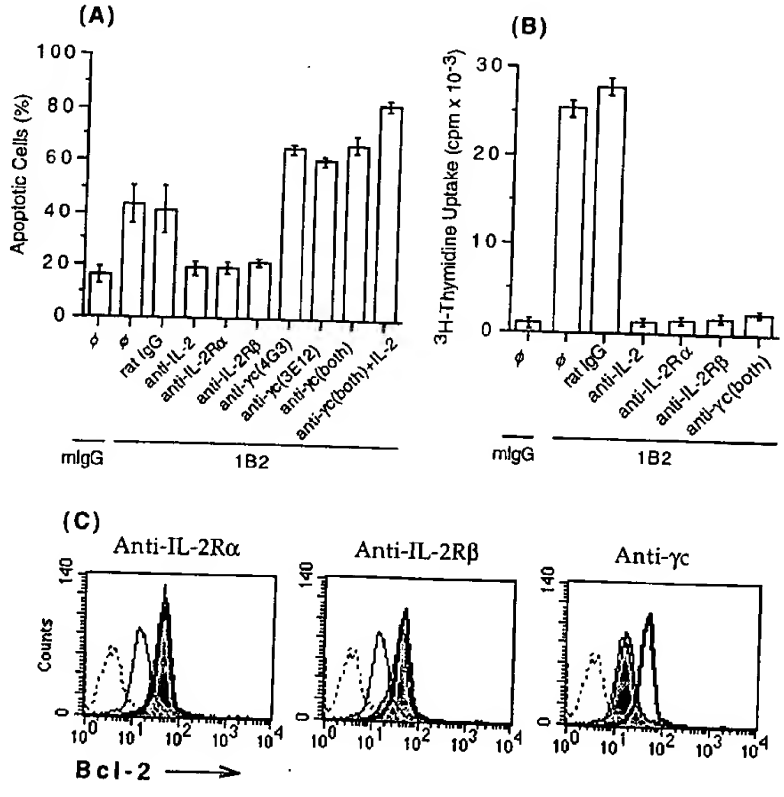

FIGURE 4. γc-blocking Abs enhance IL-2-dependent AICD of CD8$^+$ T cells and inhibit Bcl-2 induction. *A*, γc-blocking Abs enhance IL-2-dependent AICD. 2C cells were isolated from primed 2C mice and stimulated in vitro with either mIgG (control) or 1B2. Abs (10 μg/ml each) were added at the beginning of the culture. Twenty-four hours later, cells were labeled by the TUNEL method and analyzed by single-color flow cytometry. After gating on the total lymphocyte population, the proportion (percentage) of apoptotic (TUNEL$^+$) cells was calculated. ϕ, no Ab added. The mean ± SD of four experiments are shown. *B*, γc-blocking Abs inhibit 2C cell proliferation. 2C cells were isolated from primed 2C mice and cultured in plates precoated with either mIgG (control) or 1B2. mAbs to rat IgG (control), IL-2, IL-2Rα, IL-2Rβ, or γc (4G3 plus 3E12) were added (10 μg/ml each) at the beginning of the culture. [^{3}H]TdR incorporation was measured 24 h later. ϕ, no Ab added. The mean ± SD of three experiments are shown. *C*, γc-blocking Abs inhibit Bcl-2 induction. 2C cells were isolated from primed 2C mice and stimulated in vitro with either mIgG (control) or 1B2. Abs to IL-2Rα, IL-2Rβ, or γc (4G3 plus 3E12) were added (10 μg/ml each) at the beginning of the culture. Twenty-four hours later, intracellular expression of Bcl-2 was measured by single-color flow cytometry: unstained cells (dotted line), mIgG-stimulated cells (solid line), 1B2-stimulated cells (bold line), and 1B2-stimulated cells treated with the indicated Ab (shaded histogram).

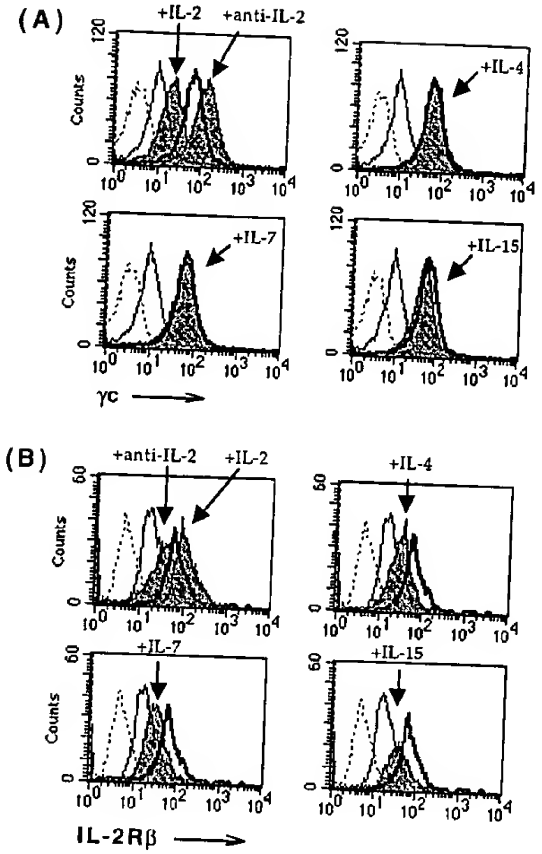

FIGURE 5. IL-2 down-regulates γc and up-regulates IL-Rβ expression on 2C cells in vitro. 2C cells were isolated from primed 2C mice and stimulated in vitro with either mIgG (control) or 1B2. IL-2 (50 U/ml), anti-IL-2 (10 μg/ml), IL-4 (20 ng/ml), IL-7 (20 ng/ml), and IL-15 (20 ng/ml) were added at the beginning of the culture. Twenty-four hours later, surface expression of γc (*A*) and IL-2Rβ (*B*) was measured by single-color flow cytometry: unstained cells (dotted line), mIgG-stimulated cells (solid line), 1B2-stimulated cells (bold line), and 1B2-stimulated cells treated with the indicated cytokine or anti-IL-2 (shaded histogram).

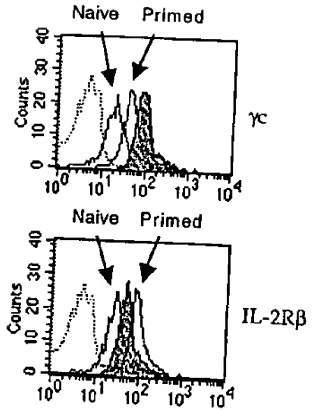

FIGURE 6. Endogenous IL-2 down-regulates γc and up-regulates IL-2Rβ expression on 2C cells in vivo. 2C mice were kept naive or were primed in the footpads with allogeneic splenocytes on days 0 and 5. Twenty-four hours later, popliteal and inguinal lymph node cells were pooled, enriched for $CD8^+1B2^+$ cells, and analyzed for γc expression (*top panel*) and IL-2Rβ expression (*bottom panel*) by single-color flow cytometry: unstained cells (dotted line) and cells from primed mice treated with 500 μg of IL-2-neutralizing Ab i.p. on day 5 (shaded histogram).

We then tested whether endogenous IL-2 regulates γc and IL-2Rβ expression in vivo. 2C mice were primed with L^d-bearing splenocytes on days 0 and 5, and their $CD8^+$ T cells were analyzed for γc and IL-2Rβ expression by flow cytometry 24 h later. As shown in Fig. 6, IL-2-neutralizing Ab given to mice at the time of secondary stimulation with allogeneic splenocytes resulted in superinduction of γc and inhibition of IL-2Rβ expression. Moreover, we found that γc expression on $CD8^+$ T cells of adult IL-2 gene-knockout ($IL\text{-}2^{-/-}$) mice was higher than that observed in wild-type ($IL\text{-}2^{+/+}$) mice (Fig. 7). Comparable levels of γc, on the other hand, were detected on $IL\text{-}2^{-/-}$ and $IL\text{-}2^{+/+}$ $CD4^+$ T cells (Fig. 7). IL-2Rβ expression on both $CD8^+$ and $CD4^+$ T cells was lower in the $IL\text{-}2^{-/-}$ group (Fig. 7). These findings indicate that down-regulation of γc expression by endogenous IL-2 may be restricted to the $CD8^+$ T cell subpopulation.

further examine the anti-apoptotic role of γc, we tested the effect of γc-blocking Abs on Bcl-2 expression. We found that anti-γc Abs, but not those that block the α- and β-chains of the IL-2R, inhibit activation-induced Bcl-2 expression in 2C cells (Fig. 4*C*).

IL-2 down-regulates γc expression on activated $CD8^+$ T cells in vitro and in vivo

Because γc is critical for the survival and proliferation of activated $CD8^+$ T cells, we asked whether IL-2 induces sensitivity to AICD by down-regulating γc expression on these cells. 2C mice were primed with L^d-bearing splenocytes. Five days later, their spleen and lymph node cells were enriched for $CD8^+1B2^+$ T cells and restimulated in vitro with plate-bound 1B2 Ab or isotype control mIgG. As shown in Fig. 5*A*, cross-linking the 2C TCR with 1B2 up-regulated γc expression. However, γc expression was significantly higher in the presence of IL-2-neutralizing Ab and was significantly lower when rIL-2 was added to the culture medium. IL-4, IL-7, and IL-15, which do not promote AICD (Fig. 1*B*), did not alter γc expression. In contrast to its effects on γc, IL-2 enhanced the expression of the IL-2R β-chain on activated 2C cells, whereas IL-4, IL-7, and IL-15 resulted in its down-regulation (Fig. 5*B*).

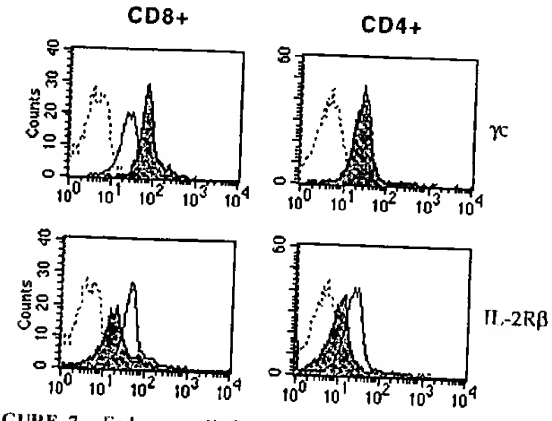

FIGURE 7. Endogenous IL-2 regulates γc expression on $CD8^+$, but not $CD4^+$, T cells in vivo. Adult $IL\text{-}2^{+/+}$ and $IL\text{-}2^{-/-}$ mice were sacrificed, and their spleen and lymph node cells were pooled and stained for CD4, CD8, γc, and IL-2Rβ surface markers. After gating on either the $CD8^+$ or $CD4^+$ cell populations, γc and IL-2Rβ expression was analyzed: unstained cells (dotted line), stained $IL\text{-}2^{+/+}$ cells (solid line), and stained $IL\text{-}2^{-/-}$ cells (shaded histogram).

Discussion

The goal of this study was to investigate the cellular mechanisms by which IL-2 prepares $CD8^+$ T cells for AICD. To do so, we established an IL-2-dependent AICD model using allospecific $CD8^+$ TCR-tg T cells. We then provided evidence that IL-2 sensitizes activated $CD8^+$ T cells to apoptosis by at least two mechanisms: 1) by up-regulating the cell surface expression of a pro-apoptotic molecule, FasL, and 2) by down-regulating the cell surface expression of γc, which is critical for the survival and proliferation of $CD8^+$ T lymphocytes.

We found that both Fas and TNFR death pathways contribute to the apoptosis of activated $CD8^+$ T cells. The roles of Fas and TNFR, however, were separated temporally. Fas contributed to early apoptosis observed within 24 h of TCR engagement, whereas TNFR prevailed as a mediator of T cell apoptosis after the first 24 h. Although Zheng et al. have shown that TNF-α-TNFR interactions mediate the AICD of most $CD8^+$ T cells, they also observed the same temporal separation between the actions of Fas and TNFR (13). Moreover, Kurts et al. provided evidence that the peripheral deletion of autoreactive $CD8^+$ T cells induced by self-Ags involves signaling through Fas (16). These data suggest that the death pathway used by an activated T cell depends on the nature and quantity of the antigenic stimulus (11, 16). We also observed that FasL-blocking Abs, TNF-α-neutralizing Abs, or both fail to suppress AICD completely in our model, suggesting that additional members of the TNFR family, for example TRAMP and TRAIL receptors (31, 32), contribute to the AICD of $CD8^+$ T cells. Importantly, we found that IL-2 up-regulates FasL expression on activated $CD8^+$ T cells within 24 h of TCR cross-linking, whereas IL-4, IL-7, and IL-15, which do not promote AICD, fail to do so. Others have shown that IL-2 up-regulates TNFR expression on $CD8^+$ T cells (30, 33). Taken together, these findings suggest that IL-2 sensitizes $CD8^+$ T cells to AICD by up-regulating the expression of cell surface molecules involved in triggering apoptosis. Increased FasL expression alone, however, is not necessarily sufficient for enhancing T cell apoptosis. Modulating the expression of intracellular molecules that either inhibit or promote Fas-mediated apoptosis is also required. These molecules include FLIP and the product of the c-*myc* protooncogene (12, 34, 35). In addition to enhancing AICD, IL-2-induced up-regulation of FasL on $CD8^+$ T cells promotes their CTL activity, because FasL-Fas is an important pathway by which CTL kill target cells (36, 37).

Alternatively, IL-2 could prepare $CD8^+$ T cells for AICD by down-regulating the expression of surface receptors that promote lymphocyte survival and proliferation. A greatly diminished amount of $CD8^+$ T cells in neonatal and adult γc gene-knockout mice suggests that γc is required for the development, survival, and proliferation of these cells (20, 23, 24). We found in this study that γc-blocking Abs increase the proportion of apoptotic cells following the induction of AICD in a $CD8^+$ TCR-tg cell population. In contrast, Abs that block the α and β subunits of the IL-2R inhibited AICD completely. Anti-γc Abs increased the proportion of apoptotic cells in our model by blocking both mitogenic and survival signals because these Abs inhibited the proliferation of activated $CD8^+$ T cells and prevented the induction of Bcl-2, an anti-apoptotic molecule. The latter finding is consistent with the markedly reduced intracellular levels of Bcl-2 in γc gene-knockout T cells (23, 38, 39). Studies demonstrating increased apoptosis of activated T cells in Bcl-2 gene-knockout mice (40), and those demonstrating a correlation between low intracellular Bcl-2 concentrations and enhanced susceptibility of CTLs to Ag-mediated apoptosis (41) provide further evidence that Bcl-2 is an important regulator of AICD.

Because γc is critical for the survival and proliferation of activated $CD8^+$ T cells, we asked in this study whether γc expression is regulated by IL-2. We found that exogenous IL-2 down-regulates γc expression in vitro and that endogenous IL-2 limits γc expression on activated $CD8^+$ T cells in vitro and in vivo. Cytokines that do not promote AICD (IL-4, IL-7, and IL-15) did not influence γc expression. These data suggest that IL-2 prepares $CD8^+$ T cells for AICD by limiting the surface expression of γc. IL-2 could regulate both transcriptional and post-transcriptional events responsible for γc production. Ohbo et al. (42) showed that IL-2 decreases the expression of a reporter gene positioned downstream of the γc promoter region. Alternatively, Noguchi et al. (43) found that γc is cleaved by calpain following activation of murine thymocytes, suggesting that IL-2 may use proteolytic pathways to reduce γc expression on T cells.

$IL\text{-}2^{-/-}$ mice develop severe lymphoproliferation characterized by the accumulation of activated $CD4^+$ and $CD8^+$ T cells, which are resistant to AICD (5, 7, 44, 45). We observed in this study that $CD8^+$ T cells from adult $IL\text{-}2^{-/-}$ mice display exaggerated γc expression. γc expression on $IL\text{-}2^{-/-}$ $CD4^+$ cells, however, was not greater than that on $IL\text{-}2^{+/+}$ lymphocytes. This observation raises the possibility that IL-2-dependent down-regulation of γc is a feedback mechanism by which activated $CD8^+$ T cells, but not activated $CD4^+$ T cells, are sensitized to apoptosis. In fact, γc-dependent signals may play a crucial role in the peripheral deletion of mature $CD4^+$ T cells, because in γc gene-knockout mice these cells accumulate over time, display activation markers, have reduced FasL expression, and are resistant to superantigen-induced elimination (20, 23, 46). In contrast, mature $CD8^+$ T cells are severely diminished in neonatal γc gene-knockout mice and do not accumulate over time (20, 24).

In summary, IL-2 ensures that the expansion of activated $CD8^+$ T cells is limited by at least two mechanisms: 1) by up-regulating pro-apoptotic molecules, such as FasL, and 2) by down-regulating γc, which provides essential mitogenic and survival signals to $CD8^+$ T lymphocytes. These homeostatic mechanisms may prevent nonspecific tissue injury following persistent viral infections and may play a role in the induction of immunologic tolerance to transplanted organs.

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Hepatocytes induce functional activation of naive CD8+ T lymphocytes but fail to promote survival

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Intraperitoneal peptide injection of TCR-transgenic mice or expression of antigen in hepatocytes leads to an accumulation in the liver of specific apoptotic CD8+ T cells expressing activation markers. To determine whether liver cells are capable of directly activating naive CD8+ T cells, we have studied the ability of purified hepatocytes to activate TCR-transgenic CD8+ T cells *in vitro*. We show that hepatocytes which do not express CD80 and CD86 co-stimulatory molecules are able to induce activation and effective proliferation of specific naive CD8+ T cells in the absence of exogenously added cytokines, a property only shared by professional antigen-presenting cells (APC). Specific T cell proliferation induced by hepatocytes was comparable in magnitude to that seen in response to dendritic cells and was independent of CD4+ T cell help or bystander professional APC co-stimulation. During the first 3 days, the same number of divisions was observed in co-cultures of CD8+ T cells with either hepatocytes or splenocytes. Both APC populations induced expression of early T cell activation markers and specific cytotoxic T lymphocyte (CTL) activity. However, in contrast to T cells activated by splenocytes, T cells activated by hepatocytes lost their cytolytic function after 3 days of co-culture. This correlated with death of activated T cells, suggesting that despite efficient activation, proliferation and transient CTL function, T cells activated by hepatocytes did not survive. Death could be prevented by adding antigen-expressing splenocytes or exogenous IL-2 to the co-culture, indicating that hepatocytes are not involved in direct killing of CD8+ T cells but rather fail to promote survival. Dying cells acquired a $CD8^{low}TCR^{low}B220^{+}$ phenotype similar to the one described for apoptotic intrahepatic T cells, suggesting an alternative model to account for the origin of these cells in the liver. The importance of these findings for the understanding of peripheral tolerance and the ability of liver grafts to be accepted is discussed.

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1 Introduction

The liver has been shown to be a site where activated antigen-specific CD8+ T cells undergoing apoptosis accumulate following injection of MHC class I-restricted TCR transgenic mice with antigenic peptides [1, 2]. These apoptotic intrahepatic T cells express lower amounts of TCR and CD8 and expressed the B220 epitope of the CD45 activation marker. Since effective activation and proliferation of naive T cells generally require interaction of CD28 with the CD80 and CD86 co-stimulatory molecules mostly expressed by dendritic cells, macrophages, monocytes and activated B cells, it has been proposed that intrahepatic apoptotic CD8+ T cells are initially activated in lymphoid organs by peptide-loaded hematopoietically derived cells. Activated T cells would then migrate to the liver and die [1, 2].

Our previous results [3] support an alternative to this "graveyard" model accounting for the origin of these intrahepatic apoptotic T cells. By using bone marrow chimeric transgenic mice expressing the alloantigen $H\text{-}2K^{b}$ on hepatocytes but not on hematopoietically derived APC, we showed that alloreactive CD8+ T lymphocytes disappeared from lymphoid organs and massively infiltrated the liver. Infiltrating cells were activated, proliferated, induced limited hepatic damage and ended up dying by apoptosis in the liver [3]. These results suggested that naive CD8+ T cells could directly be activated locally by hepatocytes without being initially primed by professional APC. This was quite unexpected since hepatocytes are not known to express CD80 or CD86.

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Abbreviations: AICD: Activation-induced cell death **CFSE:** 5-Carboxyfluorescein diacetate succinimidyl ester **PI:** Propidium iodide

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To study the ability of hepatocytes to present antigen, we established an *in vitro* system allowing us to investigate the interactions between primary hepatocytes and CD8$^+$ T cells. We show that hepatocytes are capable of inducing *ex vivo* activation and proliferation of naive CD8$^+$ T cells in the absence of CD4 help or bystander co-stimulation provided by professional APC. Hepatocyte-stimulated T cells rapidly acquired CTL function but died sooner than those activated by professional APC. This premature T cell death was not mediated by a hepatocyte dominant death signal, since it could be prevented by adding IL-2 or splenocytes to hepatocyte/T cell co-cultures. These data suggest that hepatocytes are able to induce full activation of CD8$^+$ T cells but fail to promote survival. Furthermore, we show here that dying cells activated by hepatocytes acquired a B220$^+$ TCR^{low} CD8^{low} phenotype similar to that described for apoptotic T cells detected in the liver of TCR-transgenic mice injected with specific peptides [1, 2]. Thus, our results suggest that the dying T cells found in the liver of peptide-injected mice may well have been activated directly in the liver by peptide-loaded hepatocytes. Moreover, our findings support a model where the liver plays an important role in tolerance induction, which could account for the striking ability of liver transplants to be accepted.

2 Results

2.1 Hepatocytes induce efficient proliferation of CD8$^+$ T cells in the absence of exogenously added cytokines

To study the efficiency of hepatocytes to induce proliferation of CD8$^+$ T cells, C57BL/6 hepatocytes (H-2^b) were tested in a proliferation assay for their ability to stimulate lymph node T cells from transgenic mice expressing an

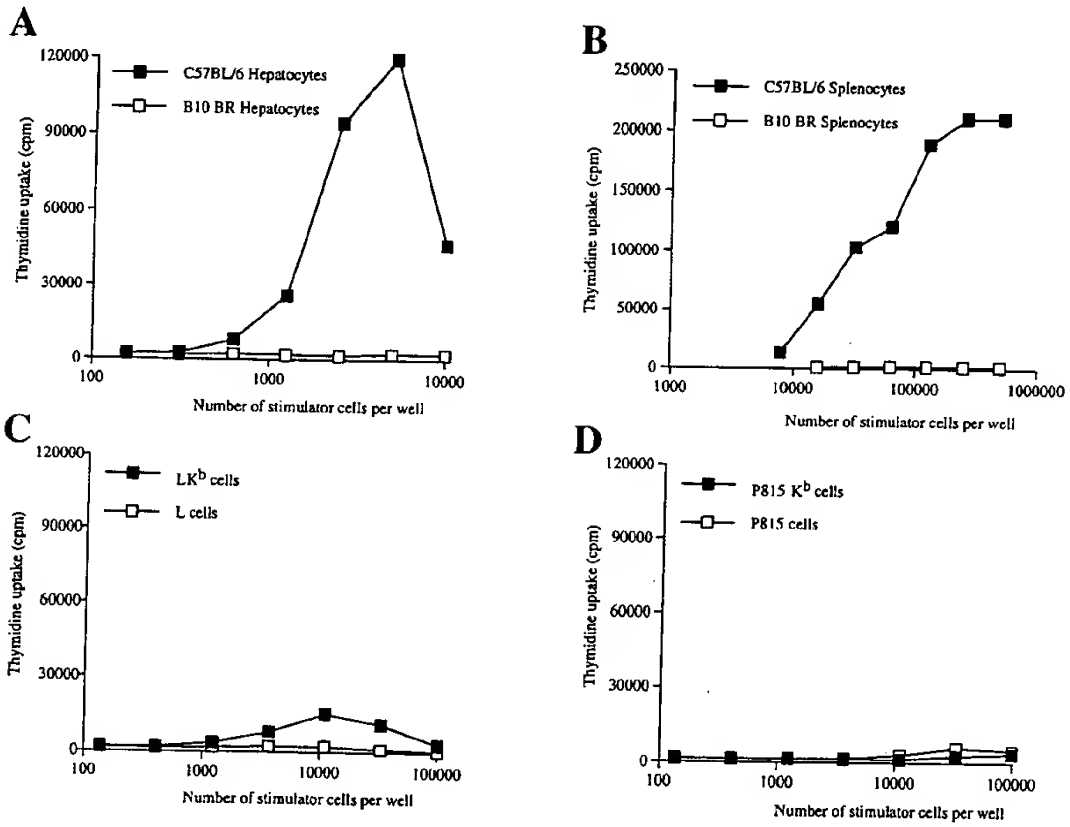

Figure 1. Hepatocytes and splenocytes induce proliferation of Des-TCR$^+$ T cells in the absence of exogenously added IL-2. Lymph node T cells from Des-TCR mice (2×10^5) were tested for their ability to proliferate after co-culture with different numbers of purified hepatocytes (A) or splenocytes (B) from C57BL/6, B10.BR control mice, fibroblastic L cells (C) or the mastocytoma P815 cell line (D) transfected or not with a cDNA coding for H-2K^b. Proliferation was measured by adding [^{3}H]thymidine for 8 h after 60 h of co-culture.

H-2K^b-specific TCR (Des-TCR mice). As shown in Fig. 1A, H-2K^b hepatocytes were able to induce proliferation of Des-TCR CD8⁺ T cells in the absence of exogenously added IL-2. Using 2×10^5 lymph node cells per well, as few as 2000 hepatocytes were required to elicit 50 % of the proliferation signal, the maximum being reached at 5000 cells per well. This response was significantly increased when exogenous IL-2 was added to the medium (data not shown). No proliferation could be detected in co-cultures of Des-TCR T cells with control H-2K^k B10.BR hepatocytes.

As expected, effective proliferation of Des-TCR T cells was observed when we used C57BL/6 splenocytes in the absence of exogenous IL-2 (Fig. 1B). However, the mouse mastocytoma P815 and mouse fibroblastic L cell lines expressing high levels of transfected H-2K^b did not promote Des-T cell proliferation (Fig. 1C and D). The inability of these cell lines to induce Des-T cell proliferation was not due the absence of cell surface MHC/peptide complexes, since strong proliferation was observed when IL-2 was added to the medium (data not shown). These results suggest that hepatocytes possess proliferation inducing properties comparable to professional APC of the spleen.

To determine the possible effects of bystander CD4⁺ T cells and/or bystander professional APC, lymph node cells from Des-TCR mice were depleted of CD4⁺, B and adherent cells. Under these conditions, proliferative responses reached levels which were comparable to those seen with non-purified T cells and which peaked

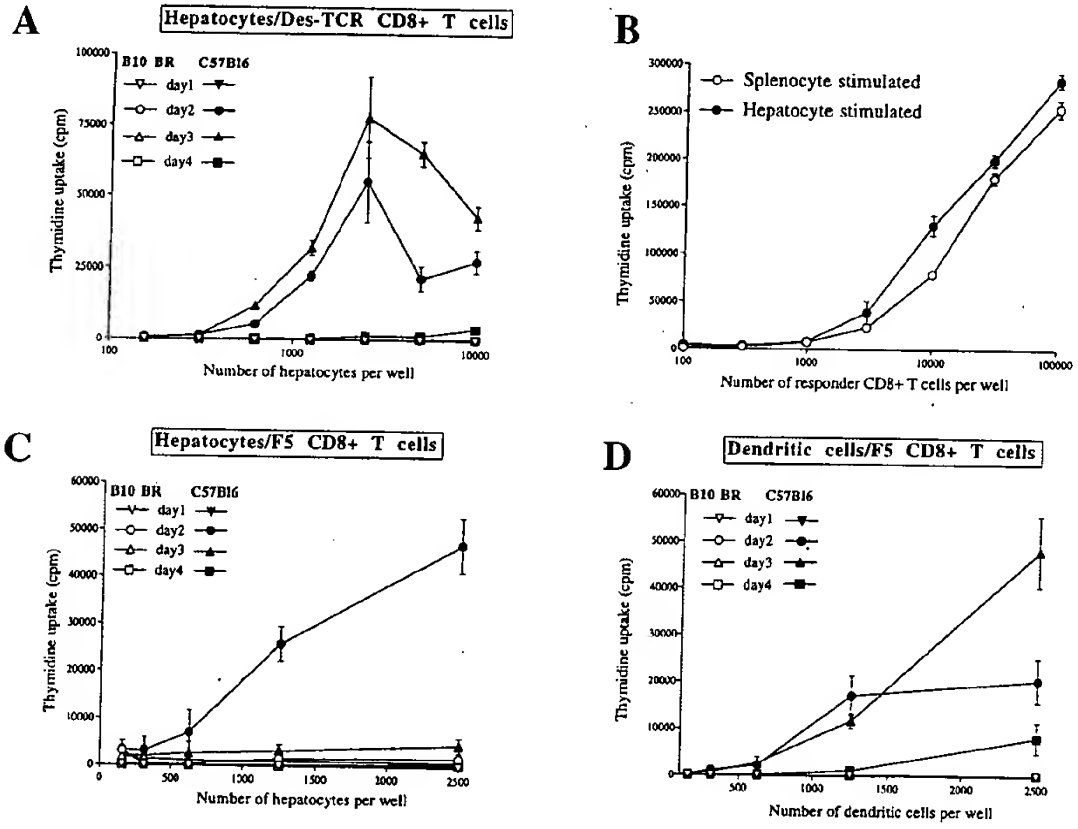

Figure 2. Hepatocytes and dendritic cells induce efficient proliferation of purified lymph node CD8⁺ T cells. Hepatocytes (A, B and C) or spleen dendritic cells (D) purified from C57BL/6 and B10.BR mice were incubated overnight at 37 °C in culture medium, and loaded (for C and D) with 1 μM NP peptide for 4 h. They were then washed and used in a proliferation assay to stimulate purified CD8⁺ lymph node T cells isolated from Des-TCR (A and B) or F5 (C and D) TCR transgenic mice. For A, C and D, we used 7×10^4 responders and different concentrations of stimulating cells and proliferation was measured by adding [^{3}H]thymidine for 8 h after different times of co-culture. For (B), dilution curves were performed by varying the concentrations of purified responder CD8⁺ T cells and by using either 5000 B6 hepatocytes or 2.5×10^5 B6 splenocytes. Proliferation was measured by adding [^{3}H]thymidine for 16 h after 2 days of co-culture.

between days 2 and 3 (Fig. 2A). In addition, these responses could be totally inhibited by adding blocking anti-CD8 antibodies to the co-cultures (data not shown). These results suggest that the proliferative responses were CD8 dependent and exclude a possible role for CD4$^+$ T cell-derived cytokines. They also rule out a contribution of bystander co-stimulation. CD8$^+$ T cells activated by saturating amounts of splenocytes or hepatocytes showed comparable levels of proliferation over a range of responder cell doses (Fig. 2B). For both types of stimulation, detectable proliferative responses were observed with as few as 1000 purified responder CD8$^+$ T cells (Fig. 2B).

To determine whether similar results would be obtained in another experimental model, we used the F5 TCR transgenic system. F5 CD8$^+$ T cells recognize the influenza nucleoprotein peptide in association with H-2D^b [4]. H-2D^b-expressing hepatocytes were loaded with 1 μM NP peptide (see Sect. 4.7) and tested for their ability to stimulate purified lymph node F5 CD8$^+$ T cells in the absence of exogenously added IL-2. Results presented in Fig. 2C show that effective proliferation was induced by hepatocytes in this different antigenic system. Unlike T cell responses seen in the Des-TCR system, F5 CD8 proliferation peaked at day 2 and failed to incorporate [^{3}H]thymidine at day 3 (Fig. 2C). This could be due either to degradation of the NP peptide *in vitro* or to a combination of the amount of MHC/peptide complexes and TCR affinity.

We also used the F5 TCR transgenic mice to compare hepatocytes to dendritic cells for their ability to induce T cell proliferation. Different numbers of freshly purified spleen dendritic cells from either H-2D^b (C57BL/6) or control H-2D^k (B10.BR) mice were loaded with 1 μM peptide and used as APC to stimulate purified lymph node F5 CD8$^+$ T cells. Despite different kinetics, maximum proliferation using dendritic cells was comparable to that obtained when using hepatocytes (Fig. 2D). Both required low numbers of stimulator cells.

Together these results show that hepatocytes are capable of inducing specific CD8$^+$ T cell proliferative responses independently of CD4$^+$ T cell help and bystander co-stimulation. In addition, these proliferative responses are as effective as those induced by professional APC.

2.2 Hepatocytes are directly responsible for T cell proliferation

To exclude the contribution of professional liver APC contaminants in hepatocyte preparations (mostly Kupffer cells and liver dendritic cells), hepatocytes were further purified by depleting H-2A^{b+} F4/80$^+$ cells with magnetic beads. Depletion was complete since RNA isolated from purified hepatocytes did not yield an H-2A^b-MHC class II band after reverse transcriptase (RT)-PCR (Fig. 3A). When tested *in vitro*, despite the decrease of the MHC class II RT-PCR band, these highly purified hepatocytes were as efficient at promoting proliferation of purified Des-TCR CD8$^+$ cells as non-purified hepatocytes

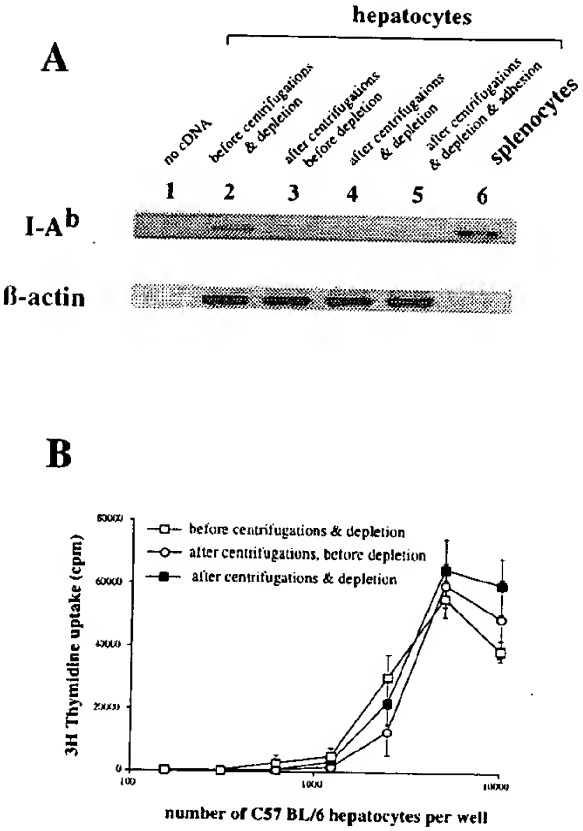

Figure 3. Hepatocytes are directly responsible for the proliferation of CD8$^+$ T cells. C57BL/6 hepatocytes were isolated to different grades of purity, just after liver perfusion without washes (step 1, lane 2), after the three usual 50 × g washes (step 2, lane 3), after step 2 followed by magnetic bead depletion of I-Ab$^+$ and F4/80$^+$ cells (step 3, lane 4) and after step 3 followed by 48 h of adhesion on plates (step 4, lane 5). (A) RT-PCR corresponding to each step of hepatocyte purification. mRNA was prepared from either splenocytes (lane 6) or 10^4 hepatocytes at each step of the purification (lanes 2 to 5), reverse transcribed and amplified using oligonucleotides specific for MHC class II I-A^b or for mouse β-actin. (B) Hepatocytes purified from steps 1, 2 and 3 were used in a proliferation assay to stimulate 7 × 10^4 purified CD8$^+$ lymph node T cells isolated from Des-TCR mice. Proliferation was measured by adding [^{3}H]thymidine for 8 h after 60 h of co-culture.

(Fig. 3B). These results suggest that CD8+ T cell proliferation can be entirely attributed to hepatocytes.

2.3 Hepatocytes are able to induce the proliferation of naive CD8+ T cells independently of CD28 co-stimulation

In the Des-TCR transgenic system, 20–25 % of the CD8+ T cell compartment expressed high levels of Ly6C and CD44. These markers correlate with a memory/activated phenotype [5, 6]. It is likely that these cells have been activated *in vivo* on environmental antigens through the second TCR resulting from the rearrangement of endogenous α chains [7]. To determine whether the naive or activated CD8+ T cell subset responds to hepatocyte stimulation, we purified CD8+ Des-TCR+ lymph node cells and sorted Ly6C− and Ly6C+ or $CD44^{low}$ and $CD44^{high}$ populations by flow cytometry. We then tested their ability to proliferate when co-cultured with C57BL/6 hepatocytes. As shown in Fig. 4A and B, hepatocytes were able to stimulate T cells of the naive phenotype. Interestingly, activated CD8+ T cells proliferated poorly under the same conditions. This could either be due to the lower avidity of these cells or to an inhibitory effect of anti-CD44 and anti-Ly6C antibodies. Similar conclusions were obtained using purified CD8+ lymph node T cells from Des-Rag-1 deficient mice which do not rearrange the endogenous α chain (Fig. 4C).

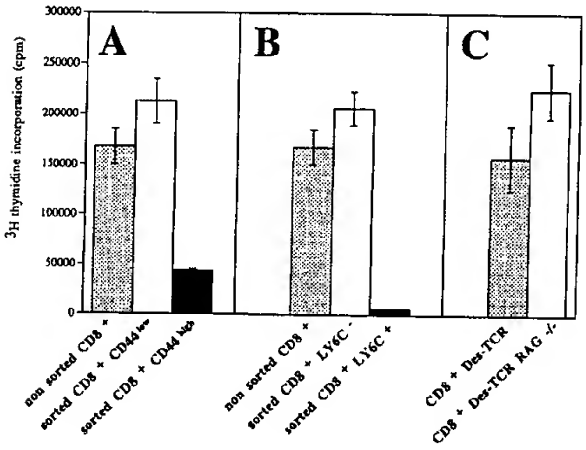

Figure 4. Hepatocytes induce efficient proliferation of naive CD8+ T cells. CD8+ lymph node T cells from Des-TCR mice (A and B) were negatively selected by magnetic bead depletion and stained with either anti-CD44 or anti-Ly6C FITC-conjugated mAb. CD44 low and CD44 high (A) or Ly6C− and Ly6C+ (B) cells were then sorted by FACS. Cells (10^5) of each population were then tested for their ability to proliferate when co-cultured with purified hepatocytes from C57BL/6 mice. (C) Proliferation assay using 2500 purified C57BL/6 hepatocytes with 5×10^4 purified CD8+ T cells from Des-TCR or Des-Rag1-deficient mice. Proliferation was measured by adding [^{3}H]thymidine for 8 h after 48 h of co-culture.

To investigate whether T cell proliferation induced by hepatocytes involves CD28 co-stimulation, purified CD8+ lymph node T cells from Des-TCR mice were co-cultured with C57BL/6 hepatocytes in the presence or absence of mCTLA4-Ig. A 5-μg/ml dose of mCTLA4-Ig was able to completely inhibit the alloresponse of purified CD8+ T cells from C57BL/6 mice towards B10.BR splenocytes (Fig. 5A). However, proliferative responses of CD8+ Des-TCR+ T cells induced by hepatocytes or splenocytes could not be blocked by using even higher concentrations of mCTLA4-Ig (Fig. 5B). Likewise, no inhibition was observed by using anti-B7.1 mAb (data not shown). These results suggest, as shown elsewhere with high avidity APC/T cell interactions [8], that the proliferative response of transgenic T cells on both hepatocytes and splenocytes is CD28 independent. As shown above, this property did not apply to other cell types expressing high levels of antigen, suggesting that, like splenocytes, hepatocytes must express other adhesion/co-stimulatory molecules allowing proliferation. One of these molecules is probably ICAM-1. Indeed, anti-ICAM-1 and anti-LFA-1 monoclonal antibodies partially inhibited the proliferative response induced by hepatocytes (Fig. 5B) suggesting that a LFA-1/ICAM-1 interaction was involved.

Collectively, these results suggest that, like splenocytes, hepatocytes can induce proliferation of naive Des-TCR CD8+ T cells independently of CD28 co-stimulation.

2.4 CD8+ T cells activated by hepatocytes or by splenocytes initially undergo the same number of divisions

To investigate whether T cells stimulated by hepatocytes or splenocytes undergo the same number of divisions, we purified CD8+ T cells from Des-TCR mice and labeled them with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) before co-culturing T cells with either C57BL/6 hepatocytes or splenocytes. CFSE is a fluorescent dye that binds covalently to cytoplasmic molecules. This labeling can be detected by FACS as a very sharp fluorescent peak whose intensity is halved with each cell division [9]. By tracking the sequential twofold decreases in fluorescence we estimated the number of divisions of activated T cells. As shown in Fig. 6, CFSE-labeled T cells activated either by hepatocytes or splenocytes undergo the same number of divisions during the first

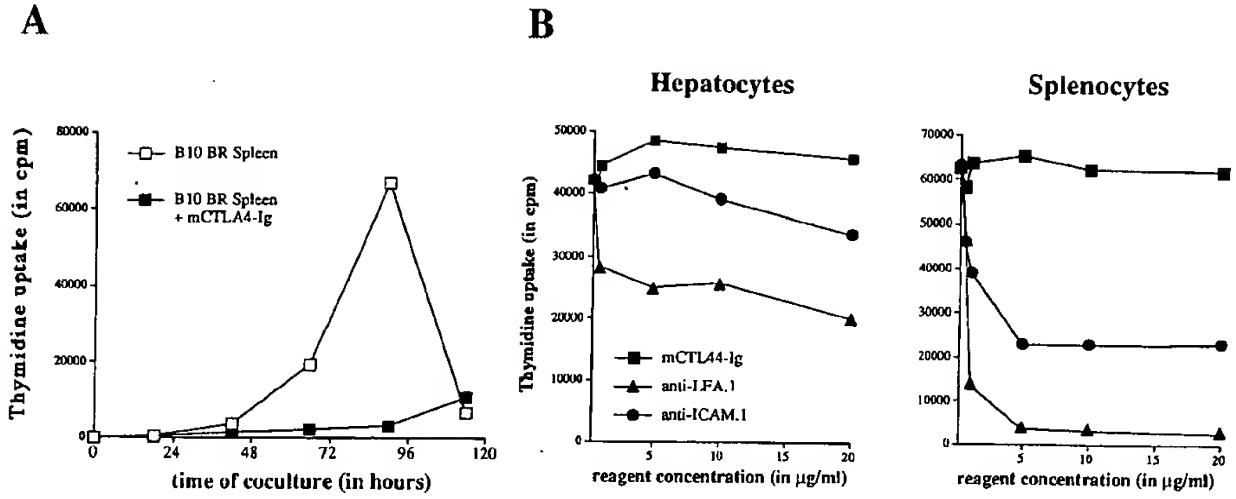

Figure 5. Hepatocyte-induced proliferation is CD28 independent. (A) As a positive control of inhibition, mCTLA4-Ig is able to inhibit most of a $CD8^+$ alloresponse. Purified $CD8^+$ lymph node cells (10^5) from C57BL/6 mice were co-cultured with 5×10^5 B10.BR splenocytes in the absence or in the presence of 5 µg/ml of mCTLA4-Ig. Proliferative responses were measured at different times by adding [^{3}H]thymidine for 8 h. (B) C57BL/6 hepatocytes (5×10^3 per well) or C57BL/6 splenocytes (1.25×10^5 per well) were preincubated for 3 h with different concentrations (in µg/ml) of either the mCTLA4-Ig reagent or anti-ICAM-1 and anti-LFA-1 purified mAb and co-cultured for 48 h with 7×10^4 of purified $CD8^+$ lymph node T cells from Des-TCR mice. Proliferation was measured by adding [^{3}H]thymidine for 8 h.

66 h. After 18 h of co-culture, some cells started expressing CD25, CD44 and CD69 early activation markers before dividing (data not shown). By 42 h of co-culture, all cells expressed these early activation markers (data not shown) and three divisions could be detected (Fig. 6). Finally, at the latest time point (66 h), some cells had already reached six divisions in both co-cultures (Fig. 6). It is interesting to note that as suggested by thymidine incorporation assays (Fig. 2), proliferation of T cells induced by hepatocytes seemed to occur slightly more rapidly than proliferation induced by splenocytes. This is also reflected by the down-regulation of early activation markers that occurs after 66 h of hepatocyte co-culture while T cells stimulated with splenocytes are still expressing these markers (data not shown).

These results suggest that $CD8^+$ T cells activated by splenocytes or hepatocytes undergo the same number of divisions and up-regulate the same early activation markers.

2.5 $CD8^+$ T cells activated by hepatocytes acquire transient CTL activity

Since hepatocytes and professional APC of the spleen share similar APC properties for the induction of naive T cell proliferation, we next investigated whether they could both induce CTL activity. Des-TCR T cells were co-cultured with C57BL/6 hepatocytes or splenocytes and tested after 2 or 3 days for their ability to lyse H-$2K^{b+}$ target cells. After 2 days, T cells activated with splenocytes were able to lyse P815-K^b specifically (Fig. 7). Slightly less potent CTL were also generated following activation with hepatocytes. However, after 3 days of co-culture, viable T cells activated by hepatocytes were no longer capable of lysing specific targets while T cells stimulated by splenocytes retained CTL function (Fig. 7). CTL function required activation since freshly isolated lymph node Des-TCR T cells were not able to lyse P815-K^b target cells (Fig. 7). These data show that T cells activated by hepatocytes proliferate and acquire CTL function, thus suggesting that they were not anergic during the early phase of the response. However although hepatocyte-activated T cells continue to proliferate, they become unresponsive at a later stage of this response. We observed that loss of cytolytic function in proliferating viable T cells activated by hepatocytes correlated with the appearance of dead cells in hepatocyte co-cultures at 66 h. We therefore investigated the fate of unresponsive T lymphocytes 66 h after activation by hepatocytes.

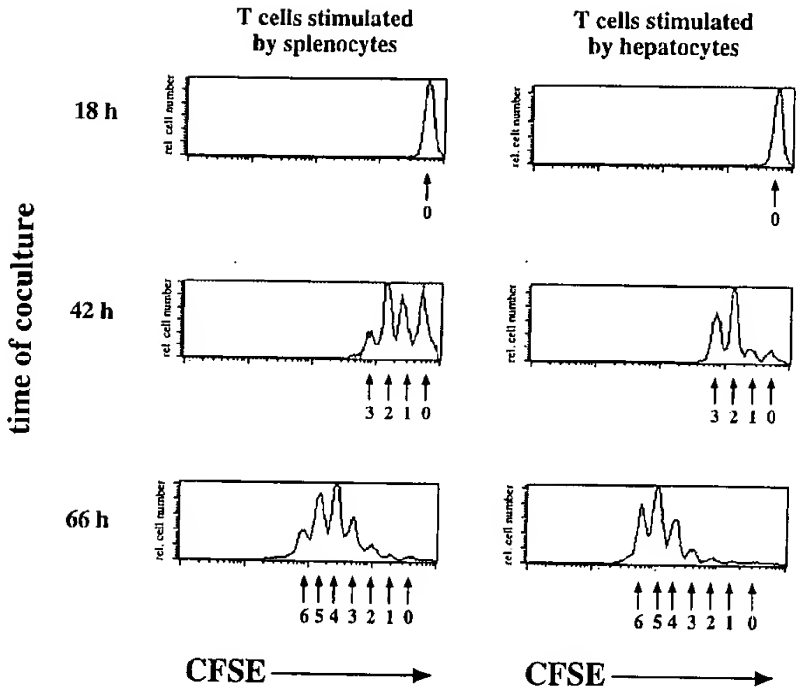

Figure 6. Number of divisions of CD8⁺ T cells activated by hepatocytes or splenocytes. Purified CD8⁺ T cells from Des-TCR mice were labeled with CFSE and added to 24-well plate tissue culture wells (5×10^5 T cells/per well) containing either 5×10^3 C57BL/6 hepatocytes or 2×10^6 C57BL/6 splenocytes. Cells were harvested at different time points and analyzed by flow cytometry for CFSE labeling by gating on $CFSE^+ PI^-$ cells.

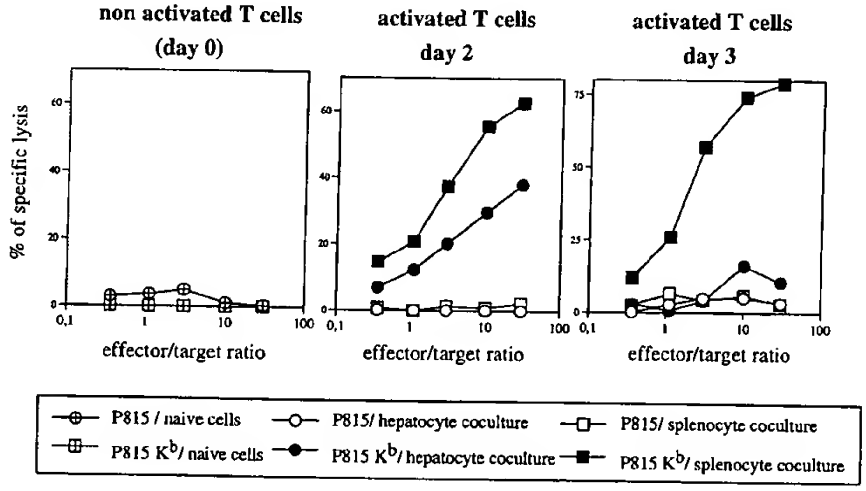

Figure 7. CTL activity of T cells stimulated by hepatocytes or splenocytes over time. Purified CD8⁺ T cells were co-cultured for 2 or 3 days with either C57BL/6 hepatocytes and splenocytes in 24-well plates as described in the legend to Fig. 6. Viable cells were Ficoll-purified and tested for their ability to lyse P815-K^b or control P815 target cells. CTL activity of non-activated T cells (freshly isolated from Des-TCR mice) towards P815 and P815-K^b target cells was also measured as a control (left panel).

2.6 CD8⁺ T cells stimulated by hepatocytes die while proliferating

To investigate the fate of T cells activated by hepatocytes, we co-cultured C57BL/6 hepatocytes or splenocytes with purified and CFSE-labeled CD8⁺ T cells from Des-TCR mice for up to 90 h. We counted the total number of specific T cells generated from each co-culture at different time points by estimating the total number of CFSE⁺ (dead and viable) cells in each well. As shown in Fig. 8A, the total number of CFSE⁺ splenocyte-stimulated T cells increased regularly over the course of the assay while the number of hepatocyte-stimulated T cells reached a plateau after 66 h in culture. As assessed by PI exclusion, the latter was due to T cell death as opposed to a block in cell division (Fig. 8A). A fraction of the T cells activated by hepatocytes started dying after 42 h, but viable cells continued dividing as previously demonstrated by CFSE labeling (Fig. 6). CFSE labeling on dead cells also revealed that T cells died irrespectively of the number of divisions (Fig. 8B). T cells died by apoptosis since they expressed phosphatidylserine on their outer membrane, as shown by annexin V binding (data not shown). Interestingly, we observed that dying cells expressed lower levels of both CD8 and transgenic Des-TCR than viable cells and induced high levels of the CD45 molecule B220 epitope (Fig. 8B) usually expressed by B cells but also reported to be a marker of intrahe-

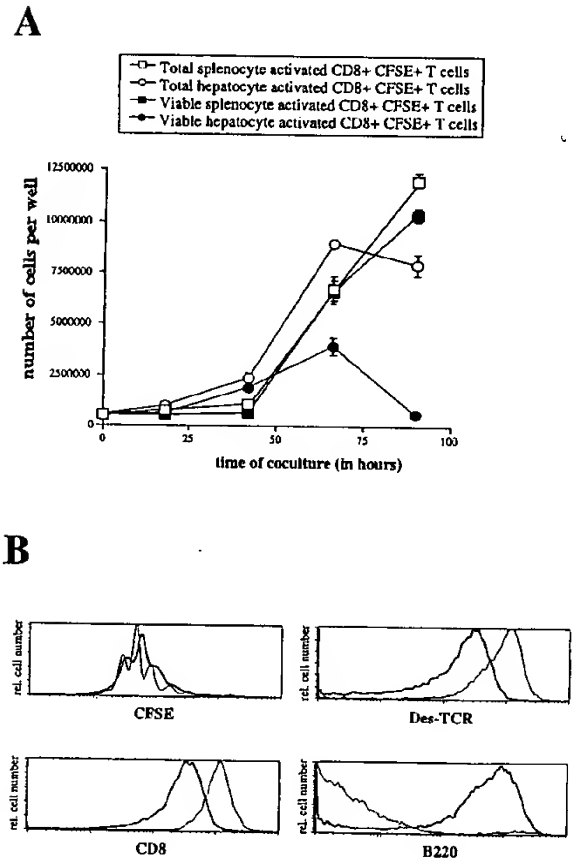

Figure 8. T cells activated by hepatocytes die prematurely. CD8⁺ T cells were purified by magnetic bead depletion, labeled with CFSE and co-cultured with C57BL/6 hepatocytes or splenocytes as described in the legend to Fig. 6. At different time points, cells were harvested, PI-labeled and analyzed by FACS. (A) Kinetics of the total number of CFSE⁺ cells and viable CFSE⁺ cells estimated by FACS analysis in splenocyte and hepatocyte co-cultures at different times. Viability was determined using PI exclusion. Each time point represents the mean value from three independent cultures. (B) Expression of CD8, Des-TCR and B220 cell surface markers in CFSE⁺PI⁻ (viable CD8⁺ T cells, thin line) and CFSE⁺PI⁺ (dead CD8⁺ T cells, full line) cells activated for 66 h with hepatocytes. CFSE levels were equivalent in both viable and apoptotic cells (upper left panel).

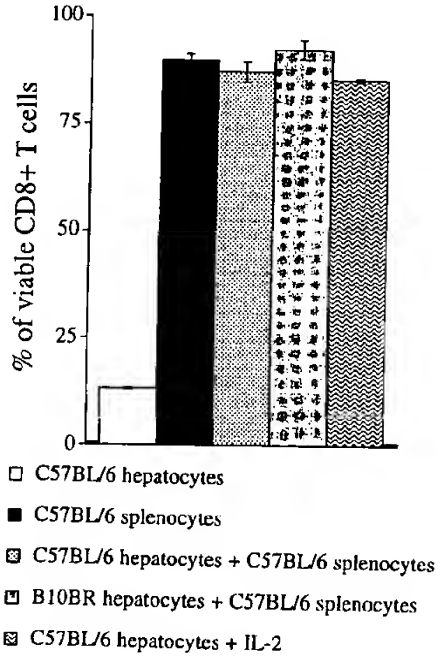

Figure 9. Premature death of CD8⁺ T cells co-cultured with hepatocytes is not due to depletion of nutrients or to a dominant death signal provided by hepatocytes. Purified lymph node CD8⁺ T cells (5×10^5) from Des-TCR mice were co-cultured for 4 days with the different cell populations indicated in the figure. Viability was estimated by PI exclusion on flow cytometry by gating on CD8⁺ Des-TCR⁻ cells. Each histogram represents the mean value from three independent cultures.

patic apoptotic CD8+ T cells [1]. This staining was specific since no staining was observed by using an isotype matched control antibody (data not shown). T cell death induced by hepatocytes was prevented for at least 4 days by adding exogenous IL-2 or stimulating H-2K^{b+} splenocytes (Fig. 9). CD8+ T cells could also survive when they were co-cultured with non-stimulating H-2K^{k+} hepatocytes and stimulating H-2K^{b+} splenocytes (Fig. 9). These results indicate that premature T cell death is not due to exhaustion of nutrients by hepatocytes and is not induced by a hepatocyte dominant death signal.

Collectively, these results suggest that hepatocytes and splenocytes initially generate similar numbers of activated CD8+ T lymphocytes, but that many of the progeny of T cells stimulated by hepatocytes die prematurely of apoptosis.

3 Discussion

By using CD8+ T cells isolated from TCR transgenic mice we have shown that hepatocytes possess the ability to induce effective *ex vivo* activation and proliferation of naive CD8+ T cells. However, this activation fails to promote long-term survival. Similar results were observed in two different MHC class I-restricted TCR transgenic mice, one expressing a TCR alloreactive against H-2K^b, the other reactive to a viral peptide presented by H-2D^b, thus generalizing our observations of hepatocyte/CD8+ T cells interactions. The observation that hepatocytes induce efficient activation, proliferation of naive CD8+ T cells and transient cytotoxic activity *in vitro* before dying appears to reflect a physiological situation. Using transgenic bone marrow chimeras expressing H-2K^b molecule on hepatocytes, we have previously shown that recent thymic CD8+ migrants expressing the Des-TCR infiltrate the liver [3]. In addition, CD8- Des-TCR+ cells present in the liver lobules are activated, proliferate and cause some liver damage before dying by apoptosis.

Several arguments indicate that T cell proliferation is induced by hepatocytes and not by the few contaminant professional APC contained in our preparation (Kupffer cells or liver dendritic cells): (i) equivalent numbers of hepatocytes and dendritic cells were required to elicit the same proliferative response, (ii) highly purified hepatocyte populations which did not contain MHC class II+ cells were still able to induce the same level of proliferation, (iii) hepatocytes were able to induce tyrosine phosphorylation of a number of cellular proteins and induced increases in intracellular calcium concentrations in a cell-cell assay (P. Dubois and P. Bertolino, unpublished data), (iv) the fate of T cells activated by hepatocytes was different from that of T cells activated by splenocytes. Furthermore, by using highly purified CD8+ T cells in our assay we excluded a possible role for CD4+-derived cytokines as well as bystander co-stimulation provided by activated B cells, dendritic cells or macrophages as described in other systems [10, 11].

The ability to induce sustained proliferation of naive T cells in the absence of exogenously added IL-2 is thought to be unique to APC able to provide co-stimulation. One of the most effective co-stimulatory pathway involves the CD28 molecule which interacts with its natural APC ligands CD80 and CD86. CD28 co-stimulation has been shown to act synergistically with the signals provided by the TCR [12–14]. It promotes efficient T cell activation and proliferation and it increases IL-2 production by regulating both transcription of the IL-2 gene and stability of IL-2 mRNA [13, 15–17].

Unlike professional APC, hepatocytes do not express CD80 or CD86 molecules as shown by flow cytometry (data not shown). Furthermore, we showed that T cell proliferation induced by hepatocytes was not inhibited in the presence of mCTLA4-Ig, suggesting that it was independent of CD28 co-stimulation. T cell responses in the absence of CD28 co-stimulation on non-professional APC were shown to require high antigen doses and/or high avidity TCR [8, 18, 19]. Hepatocytes expressed lower densities of cell surface H-2K^b molecules than C57BL/6 splenocytes as assessed by flow cytometry (approximately 15 times less, data not shown), suggesting that we are not working in a high antigen dose range. It is probable that the transgenic TCR is a high affinity receptor. However, this alone cannot explain the ability of hepatocytes to induce effective proliferation, since under similar conditions CD8+ T cells expressing the same TCR proliferated poorly following activation by P815-K^b or L-K^b cells which express higher densities of H-2K^b molecules on their surface (similar to the density detected on splenocytes, data not shown). This suggests that hepatocytes can induce effective proliferation in the absence of CD28 co-stimulation despite low MHC expression and a TCR avidity that is uneffective when using other H-2K^{b+} cells. This property is only shared by professional APC of the spleen as shown in CD28$^{-/-}$ mice and use of mCTLA4-Ig in CD8 responses (this report and [8, 20, 21]. It has been shown that CD8+ T cells from CD28$^{-/-}$ mice can clear some viruses, but this response was restricted to viruses such as LCMV, which replicate widely and for a long period of time before being cleared [20, 22, 23]. These results did not apply to less virulent viruses, suggesting that activation in the absence of CD28 co-stimulation requires continuous triggering of the TCR with prolonged antigen exposure [20]. In this context, it is therefore possible that due to their very large size and flat

adherent shape, hepatocytes have physical properties that favor permanent interaction of MHC/peptide complexes with TCR molecules. It is also possible that in the absence of CD28 co-stimulation, adhesion molecules expressed by hepatocytes provide substitute co-stimulatory signals thus promoting effective proliferation. Interaction between LFA-1 and ICAM-1 molecules has been shown to be important for inducing T cell proliferation in the absence of CD28/B7 interaction [24]. In support of this model, we were able to partially inhibit hepatocyte-mediated proliferation using anti-LFA-1 or anti-ICAM-1 antibodies. This would suggest that hepatocytes induce proliferation of $CD8^+$ T cells through a signal involving the TCR, LFA-1 and other co-stimulatory or adhesion molecules. Despite the lack of CD28 co-stimulation, T cells activated by hepatocytes induce CD25 expression and appear to produce significant amounts of IL-2 during the early phase of the response (data not shown). This suggests that an interaction of IL-2 with its receptor could occur and play a role in the proliferative response induced by hepatocytes.

Despite the fact that T cells activated by hepatocytes or splenocytes underwent the same number of divisions, became functional and expressed the same early activation markers during the first 66 h of co-culture, their fates were different. T cells activated by hepatocytes eventually die in culture, irrespective of the number of divisions. Engagement of death mechanisms is most probably responsible for the loss of CTL activity after 66 h of co-culture. Activation-induced cell death (AICD) of T cells seems to be a general but late mechanism which participates in maintaining homeostasis of the T cell pool after an immune response. AICD of $CD4^+$ T cells has been observed after activation and proliferation in response to superantigens [25] or specific antigens [26] and is mediated by Fas. AICD of $CD8^+$ T cells has also been reported [27, 28]. However, unlike $CD4^+$ T cells, AICD of $CD8^+$ T cells seems to be mediated by the p75 TNF receptor rather than by Fas [29]. In both cases, AICD seems to be a late phenomenon that occurs after T cell activation by professional APC expressing CD80 and CD86 [26, 27]. Since T cell death mediated by hepatocytes occurs much sooner than that seen in splenocyte co-cultures, it is likely that the mechanisms involved in the induction of the "classical" AICD induced by professional APC and those involved in the premature T cell death induced by hepatocytes are different. Although they are unknown, the signaling events inducing hepatocyte-mediated T cell death do not seem to be triggered by dominant death signals like Fas ligand, TNFR or other death molecules since $CD8^+$ T cells co-cultured with stimulating hepatocytes and splenocytes survived as well as T cells co-cultured only with splenocytes.

Therefore, premature death induced by hepatocytes more probably results from the lack of expression of a survival signal. CD28 co-stimulation, as well as signal transduction through the IL-2Rγ chain, which belongs to several cytokine receptors, including IL-2, IL-4, IL-7 and IL-15, do indeed induce *bcl-*x_L and/or *bcl-2* survival genes [30–32], accounting for the important role of these cytokines in sustaining T cell responses. $CD8^+$ T cells from both $CD28^{-/-}$ or $CD28^{+/+}$ mice have been shown to proliferate effectively at early time points *in vitro* and exhibit CTL activity [20]. However, proliferative responses of $CD8^+$ T cells activated in the absence of CD28 co-stimulatory signals peak earlier [8, 18, 21], do not induce the *bcl-*x_L survival gene and die prematurely [21]. Similar observations have been reported for naive $CD4^+$ T cells [26]. We showed here that T cell proliferative responses induced by hepatocytes peaked earlier than proliferative responses induced by splenocytes or dendritic cells. We therefore favor the possibility that despite receiving the signals required for effective initial proliferation and cytotoxic function, T cells activated by hepatocytes die prematurely because they fail to receive the CD28 co-signal mediating the expression of survival genes and/or to promote efficient and sustained IL-2 production. Our results and the observations reported in the $CD28^{-/-}$ system suggest that activation/proliferation and survival of T cells are distinct mechanisms which can be dissociated. In this context, hepatocytes may provide an alternative and perhaps more physiological system for investigating these mechanisms.

This study suggests that hepatocytes behave like a new type of APC. Like classical "professional APC", they are capable of inducing T cell activation, proliferation and cytolytic activity. However, unlike professional APC, the premature T cell death due to activation by hepatocytes would result in tolerance instead of immunological memory. This APC property of hepatocytes could play a major role in inducing peripheral tolerance by deleting autoreactive $CD8^+$ T cells circulating through the liver. The anatomy of the liver is compatible with this hypothesis. Indeed, unlike most organs that possess an endothelial barrier impeding $CD8^+$ T cell access to organs unless activated, the liver possesses a unique fenestrated endothelium that allows blood cells to enter the sinusoids and contact hepatocytes [33–36]. Naive $CD8^+$ T cells could, therefore, have easy access to hepatocytes, and if they are autoreactive, could be tolerized. In agreement with this hypothesis, we previously showed that new Des-TCR^+ $CD8^+$ thymic emigrants produced in chimeric transgenic mice expressing $H\text{-}2K^b$ on hepatocytes but not on hematopoietic cells infiltrated the liver, proliferated but were eventually deleted [3]. Moreover, these mice were tolerant to $H\text{-}2K^b$ since they were unable to reject skin grafts expressing this antigen (P.

Bertolino and W. Heath, unpublished data). It would be interesting to know whether such tolerizing properties are restricted to hepatocytes or whether they can also be observed in primary cells from other tissues like the kidney and pancreas that have extremely effective vascular barriers precluding T cell access [35].

Our results could also account for the presence of apoptotic CD8+ T cells in the liver of MHC class I-restricted TCR transgenic mice injected with peptides [1, 2]. Unlike peripheral CD4+ and CD8+ T cells these intrahepatic CD8+ T cells were shown to express the B220 epitope of the CD45 molecule and down-regulated cell surface levels of both CD8 and TCR [1]. From these results, it was proposed that T cells activated in the periphery on peptide-loaded professional APC and undergoing apoptosis acquired a $B220^+CD8^{low}TCR^{low}$ phenotype and migrated to the liver to be eliminated [1]. Our results support an alternative model where intrahepatic CD8+ T cells have been initially activated *in situ* by peptide-loaded hepatocytes, inducing their proliferation and their death in the liver. It is interesting to note that dying CD8+ T cells stimulated by hepatocytes were also $B220^-CD8^{low}TCR^{low}$. This phenotype does not seem to be restricted to intrahepatic apoptotic T cells, but rather appears to be a general feature of T cells undergoing apoptosis. Indeed, T cells activated by superantigen *in vivo*, especially those with a low forward scatter profile characteristic of apoptotic cells, were also shown to express the B220 epitope and reduced amounts of TCR [37]. In our system, we observed similarly that PI^+CD8^+ T cells (those with a low forward scatter profile) activated *in vitro* by splenocytes were $B220^+CD8^{low}TCR^{low}$.

We suggest that by deleting harmful autoreactive CD8+ T cells, hepatocytes could be the main tolerogenic cells involved in the striking ability of liver transplants to be accepted. Indeed, the transplantation of most organs between individuals of a species usually results in rejection of the graft unless the donor and the recipient express the same MHC molecules. Liver transplants appear to be the only exceptions to this rule, since they are rarely rejected, even if the recipient is not MHC compatible [38]. Moreover, grafting a liver between allo-MHC individuals induces specific tolerance to other transplants that would otherwise be rejected [39]. How this tolerance is achieved remains poorly understood. Chimerism of recipient tissues with donor hematopoietic cells (also called "passenger leukocytes") is often correlated with graft acceptance [40, 41]. The current model to explain the unique ability of liver transplants to induce tolerance is that due to its large size, the liver contains high numbers of passenger leukocytes and/or its progenitors [40]. These hematopoietic cells would migrate to the host thymus and lymphoid organs and establish long-term tolerance by deleting alloreactive CD8+ T lymphocytes [42]. Although chimerism is often observed in transplanted recipients, it is not known whether it is the cause or a consequence of tolerance [40]. Our results suggest that by deleting alloreactive CD8+ T cells, hepatocytes could contribute to the induction of tolerance.

In conclusion, our results provide the first evidence that hepatocytes can induce full activation of T cells leading to death instead of survival. This property of hepatocytes could play a major role in establishing peripheral tolerance and acceptance of liver transplants.

4 Materials and methods

4.1 Mice

Transgenic mice, B10.BR ($H\text{-}2^k$) and C57BL/6 ($H\text{-}2^b$) mice were bred at the Ecole Normale Supérieure de Lyon. "Des-TCR"-transgenic mice expressing a K^b-specific TCR [43] identifiable by a clonotypic antibody, "Désirè" [44], were kindly provided by Drs. B. Arnold, G. Schönrich and G. J. Hämmerling (German Cancer Research Center, Heidelberg, Germany). The Des-TCR line was inbred by mating with B10.BR mice and the progeny screened by flow cytometry. Rag-1-deficient Des-TCR mice were kindly provided by Dr. William Heath (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and inbred in germ-free conditions in our animal facilities. F5 transgenic mice expressing a TCR specific for the peptide 366–374 of influenza virus nucleoprotein [4] were a kind gift of Dr. Dimitri Kioussis to Maria Pihlgren who provided mice from her colony established at the ENS animal house.

4.2 Cell lines and cell culture

The L-K^b and P815-K^b cell lines were grown in DMEM culture medium (Life Technologies, France) supplemented with 2 mM L-glutamine (Life Technologies, France), 40 µg/ml gentamycin (Life Technologies), 6 % FCS (TechGen, France), 10 mM Hepes and 50 µM 2-mercaptoethanol at 37 °C and 7 % CO_2.

All proliferation assays were done in RPMI 1629 culture medium supplemented with 2 mM L-glutamine (Life Technologies) 40 µg/ml gentamycin (Life Technologies), 6 % FCS (TechGen), 10 mM Hepes and 50 µM 2-mercaptoethanol. Assays were performed in 96-well flatbottom plates at 37 °C and 7 % CO_2. This medium will subsequently be referred to as RPMI 1629/10 % FSC.

4.3 Cell preparation

Lymph nodes and spleen were removed and transferred into supplemented DMEM containing 6% FCS, and cell suspensions were made by pressing the tissue through a wire mesh and washing twice with cold balanced salt solution (BSS). Hepatocytes were isolated according to a modification of the classic procedure described by Seglen et al. [45]. Livers were retrogradely perfused via the inferior vena cava using Hanks' BSS (HBSS) without calcium and magnesium, and with the same medium containing 0.5 mM EDTA. EDTA was flushed out using HBSS medium without calcium and magnesium. Hepatocytes were released by continuing the perfusion with HBSS without magnesium containing 5 mM $CaCl_2$ and 0.05% collagenase IV (Sigma, St. Louis, MO; ref. C-5138). The reticular endothelial system was removed. The cells were washed four times by 50 × g centrifugation and counted.

4.4 Antibodies

For flow cytometry stainings, we used anti-mouse CD8 (YTS 169, [46]) coupled to FITC, anti-TCR clonotypic Désiré ([44], kindly provided by Dr. A. M. Schmitt-Verhulst, Marseille Luminy), anti-mouse CD44 (IM7.81, [47]), anti-mouse CD25 (CL8925B, Cedarlane Laboratories) and anti-mouse CD69 (HI.2F3, Pharmingen, San Diego, CA) biotinylated mAb. Streptavidin-PE were purchased from Caltag Labs, anti-B220-PE (RA36B2) from Sigma, FITC-labeled anti-H-$2A^b$ (25-9-17) from Pharmingen (San Diego, CA). The anti-mouse CD11c (N418) coupled to FITC was kindly provided by Dr. Muriel Moser. The blocking mCTLA4-Ig chimeric protein [48] was a kind gift from Dr. Peter Lane. The anti-ICAM-1 (YN1/1.7, [49]) and anti-LFA-1 (FD441.8, [50]) mAb were purified on G protein coupled to Sepharose beads. For magnetic bead depletion, we used hybridomas supernatants producing anti-mouse CD4 (GK1.5, [51]), anti-B220 (RA36B2, [52]), macrophage-specific anti-F4/80 (F4/80, [53]) and anti-H-$2A^b$ (M5/114, [54]) mAb. To sort activated and naive $CD8^+$ $Des\text{-}TCR^-$ T cells by FACS we used anti-mouse CD44 (IM7.81, [47]) or anti-Ly6C (143.4.2, [6]) coupled to FITC.

4.5 Purification of spleen dendritic cells

Spleen dendritic cells were purified according to the classical technique described by Metlay et al. [55]. Briefly, spleens from ten C57BL/6 or B10.BR control mice were perfused with HBSS medium without calcium and magnesium containing 100 U/ml collagenase D. The perfusate was collected and perfused spleens were further incubated for 15 min at 37 °C with HBSS medium containing 400 U/ml of collagenase D. Digested spleens were then passed through 100-μm wire mesh sieves and the cell suspension added to the perfusate. Cells were washed with supplemented DMEM medium and the pellet resuspended in 5 ml of BSA. The cell preparation containing mostly macrophages and dendritic cells was centrifuged for 20 min at 7500 rpm and 4 °C and the interphase collected. These cells were washed and incubated for 2 h in petri dishes at 37 °C in fresh medium. Petri dishes were then washed extensively to remove all nonadherent cells, refilled with fresh medium and incubated for 18 h at 37 °C to allow dendritic cells to detach from the plastic. Nonadherent cells containing mostly dendritic cells were then collected and counted. The cells collected by this technique were mostly dendritic cells since 90% of them were MHC class II$^+$ and $N418^+$ cells as checked by flow cytometry analysis.

4.6 Magnetic bead depletion

Lymph node $CD8^+$ T cells were purified by magnetic beads using a negative selection strategy. Briefly, lymph node cells isolated as described above were incubated for 2 h at 37 °C and 7% CO_2 in RPMI 1629/10% FCS medium in petri dishes to remove adherent cells. Nonadherent cells were then carefully harvested and incubated for 30 min at 4 °C with a mixture of supernatants containing anti-mouse CD4 (GK1.5) and anti-B220 (RA36B2) mAb. Cells were washed three times and incubated for 30 min at 4 °C with magnetic beads coupled with goat anti-rat IgG (H & L) (Biomag®, PerSeptive Diagnostics, GB) at a ratio of ten beads per cell. Cells attached to the beads were removed by a magnet and were washed once before being added to the plate. The cell preparation obtained using this protocol contains usually high and reproducible yields of $CD8^+$ cells (between 90% and 98%) as checked by flow cytometry analysis. The same depletion protocol was used to remove H-$2A^{b+}$ and $F4/80^+$ T cells from the hepatocyte preparation but the cells were incubated with supernatants containing anti-H-$2A^b$ (M5.114) and anti-F4/80 (F4/80) mAb instead of anti-CD4 and anti-B220 mAb.

4.7 Peptide and peptide loading protocol

The peptide recognized by the F5 transgenic TCR is the A/N/T/60/68 influenza virus nucleoprotein peptide Ala-Ser-Asn-Glu-Asn-Met-Asp-Ala-Met [NP-(366–374)]. The synthetic peptide (purchased from Neosystems Laboratoire) was dissolved in PBS.

To load hepatocytes with different concentrations of peptide, hepatocytes were prepared and incubated overnight in 96-well flat-bottom plates in RPMI 1629/10% FCS. Adherent hepatocytes were then incubated for 3–4 h with the appropriate concentration of peptide in the same medium and washed three times in the plate to remove peptide contained in the supernatant. Purified $CD8^+$ T cells from F5 mice were added just after the last wash.

To load dendritic cells and splenocytes, a similar loading protocol was used, except that splenocytes were isolated the same day they are loaded and that peptide incubation and washes were performed in tubes rather than plates. Cells were then counted and added to 96-well flat-bottom plates before adding the T cells.

4.8 CFSE labeling

CFSE was purchased from Molecular Probes (Interchim, France) and used to label cells as described elsewhere [9]. $CD8^-$ T cells (10^6 to 5×10^7 per ml) isolated from lymph nodes of Des-TCR mice were purified by negative selection with magnetic beads as described above. For CFSE staining, purified $CD8^+$ T cells were incubated for 10 min at 37 °C with 10 μM of CFSE in RPMI 1629/10% FCS. Cells were washed twice before co-culturing them with hepatocytes or splenocytes.

4.9 Flow cytometry

Cells (10^6 per sample) were stained first with biotin-coupled antibodies for 40 min in plates. After three washes, cells were stained with streptavidin-PE and FITC-conjugated antibodies for a further 40 min, washed three times, and analyzed. PI was also added at 1 μg/ml to exclude dead cells. All incubations above were performed on ice in the dark. Analysis was performed on a FACScan® (Becton Dickinson and Co.). Live gates were set on lymphocytes by forward and side scatter profiles and 10^4 PI-negative cells were analyzed.

4.10 Proliferation assay

Purified $CD8^-$ lymph node responder cells from Des-TCR or F5 mice (5×10^4 to 10^5) were incubated in RPMI 1629/10% FCS with different concentrations of stimulator cells for 60 h at 37 °C in 96-well plates. Before adding them to culture, dendritic cells and splenocytes were irradiated at 1500 rad, whereas L, L-K^b, P815 and P815-K^b cells were irradiated at 10000 rad. Since irradiation has no effect on the ability of hepatocytes to induce proliferation of $CD8^+$ T cells, all the experiments shown in this study was performed with non-irradiated hepatocytes. Hepatocytes were purified the day before as described above and incubated overnight in the plates before adding the T cells. Proliferation was measured by adding 10 μCi of [^{3}H]thymidine in each well and thymidine incorporation was allowed to proceed for 8 h at 37 °C. Insoluble material was harvested and counted.

4.11 Cytotoxic assay

Purified $CD8^+$ lymph node cells from Des-TCR mice (5×10^5) were co-cultured in RPMI 1629/10% FCS at 37 °C in 24 well plates with either 2×10^6 1500-rad irradiated C57BL/6 splenocytes or 5×10^4 C57BL/6 adherent hepatocytes. Hepatocytes were purified the day before as described above and incubated overnight in 24-well plates before adding the T cells. After 2 or 3 days of co-culture, effector cells were purified over Ficoll, and seeded onto 96-well round-bottom plates. Target cells (10^6 P815 and P815-K^b cells) were incubated with 50 μCi of ^{51}Cr for 1 h at 37 °C. Target cells (3000/well) were added to the different concentrations of effector cells and incubated for 4 h at 37 °C. Assays were done in duplicates. Counting was done with Microbeta Trilux (Wallac).

4.12 RNA purification

RNA for PCR was prepared from 10^4 hepatocytes depleted or non-depleted of I-A^{b-} and F4/80$^+$ cells. Cells were pelleted and treated with 800 μl of RNA Now (BioGentex Inc.). Samples received 400 μl chloroform and were centrifuged for 15 min at 10000 rpm and 4 °C. The upper phase was then mixed with 400 μl of isopropanol and 10 μg of glycogen, centrifuged for 10 min at 10000 rpm. The pellet containing RNA was washed once with ethanol and air dried.

4.13 Reverse transcription and cDNA amplification

RNA samples were transcribed with reverse transcriptase and cDNA were amplified by PCR in 100 μl, using the GeneAMP RNA PCR kit (Perkin-Elmer Cetus Instruments, Norwalk, CT), according to the manufacturer's recommendations. Ampfliciations were performed in the GeneAmp PCR System 9600 thermalcycler (Perkin Elmer, Cetus Instruments) under the following conditions: 94 °C, 3 min; 40 cycles of 94 °C, 1 min; 55 °C, 2 min; 72 °C, 3 min; 72 °C, 10 min. PCR products were examined by ethidium bromide staining after electrophoresis.

4.14 Primers

PCR were primed with oligonucleotides specific for H-2A^b β chain and mouse β-actin cDNA. H-2A^b β chain-specific primers were H-2A^b β 7081 complementary to residues 7081–7100 in exon 4 (5'-TGACTCC-TGTGACGGATGAA-3') and sense-strand primer H-2A^b β 6636, corresponding to residues 6636–6655 in exon 3 (5'-ACACCTGTCACGTGGAGCAT-3') [56]. Mouse β-actin specific primers were β actin 1563 complementary to residues 1563–1586 in exon (5'-GCTTTTGGG-AGGGTGAGGGACTTC-3') and sense-strand primer β actin 692, corresponding to residues 692–715 in exon (5'-TGAGAGGGAAATCGTGCGTGACAT-3').

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The 4F9 Antigen Is a Member of the Tetra Spans Transmembrane Protein Family and Functions as an Accessory Molecule in T Cell Activation and Adhesion[1]

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In this report, we describe a 43- to 50-kDa protein, which may function as a costimulatory molecule for full activation of human T cells. This Ag, defined by a mouse monoclonal antibody (mAb) anti-4F9, is primarily distributed on "helper/inducer" or "memory" $CD4^+CD45RO^+$ subset. Like mAbs against many other accessory/costimulatory molecules, coimmobilization of anti-4F9 with anti-CD3 resulted in synergistic T cell proliferation. In addition, immobilized anti-4F9 on plastic plates induced T cell spreading characterized by the development of prominent dendritic processes. A cDNA encoding the 4F9 Ag was isolated from a cDNA library constructed from PHA/PMA-activated T cells using a COS cell expression system. The sequence of the cDNA and a homology search revealed that the 4F9 Ag was identical to R2, a molecule recently cloned by subtractive hybridization. The 4F9/R2 Ag belongs to a newly identified supergene family (tetra spans transmembrane protein family) characterized by four putative transmembrane domains which are highly conserved between the members of this family. Based upon the phenotypical and functional studies described here, we propose that the 4F9 Ag is an integral membrane protein which can transmit signals involved in T cell proliferation and adhesion. The preferential distribution of this molecule on the $CD4^+CD45RO^+$ subset of T cells may contribute to the distinct activation profile and functional repertoire of these cells.

INTRODUCTION

While essential for initiation of T cell activation, the interaction between the CD3/TcR and Ag/MHC on APC is insufficient to complete the proliferative response and generate functional programs of T cells (1–3). Participation of additional cell-surface molecules (accessory molecules) that mediate adhesion and/or influence signal transduction is required for optimal T cell activation. These molecules were initially defined and characterized by the use of specific monoclonal antibodies (mAbs) capable of blocking or enhancing functional T cell responses. Many such accessory molecules have now been shown to be adhesion structures belonging to the Ig gene superfamily or the integrin gene family (4). These include CD2, CD4, CD8, CD28, CD11a/CD18 (LFA-1), and the VLA/CD29 Ags (5–9). Most of these molecules appear to regulate

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T cell activation in conjunction with the TcR/CD3 complex by facilitating adhesive interactions of T cells with APCs or target cells and by delivering costimulatory activation signals to the T cell. In addition, other non-Ig, nonintegrin molecules, including CD26, CD44, and CD45, also play significant roles in T cell function (10–12).

Importantly, some accessory molecules are differentially expressed on T cell subsets. For example, the majority of mature T cells express either CD4 or CD8 (6), thus defining the two main subsets of T cells capable of interacting with MHC class II and MHC class I, respectively. $CD4^+$ cells can be further subdivided into two reciprocal subsets depending upon differential expression of CD45 (leukocyte common antigens) isoforms (12). $CD45RA^+CD45RO^-CD4^+$ cells have been characterized as "naive" and/or "suppressor/inducer" cells, while $CD45RA^-CD45RO^+CD4^+$ cells have "memory" and/or "helper/inducer" phenotypes (13–16). In addition, several other adhesion/accessory molecules are preferentially expressed on $CD4^+$ cells bearing the CD45RO isoform (4). Thus, differential expression of multiple accessory molecules may contribute to the selective activation of distinctive T cell subsets, thereby promoting the subsequent diverse functional responses.

In the present paper, we describe another molecule primarily expressed on the $CD45RA^-CD45RO^+$ CD4 subset of resting T cells. This Ag, defined by mAb anti-4F9, was identical to the R2 Ag described previously (17), which belongs to a newly identified supergene family (tetra spans transmembrane protein family) characterized by a structure having four distinct transmembrane domains (17–19). Using anti-4F9 mAb, we attempted to determine functional roles of this unique Ag in T cell immune function.

MATERIALS AND METHODS

mAbs and Preparation of Cells

mAbs reactive with CD1 (T6), CD3 (RW24B6), CD4 (19Thy5D7), CD8 (21Thy2D3), CD11a/LFA-1 (2F12), CD20 (B1), CD29/VLA-β1 (4B4), CD31 (1F11), CD45 (GAP 8.3), CD45RA (2H4), CD45RO (UCHL1), CD56 (NKH1), L-selectin (TQ1), MHC class I (W6/32), and MHC class II (9-49) were used in our study. Their production and characterization have been described elsewhere (13–15, 20–22). PBLs obtained from healthy donors were separated into E rosette-positive (T cells) and -negative populations with SRBC. The T cells were depleted of contaminating monocytes by adherence to plastic plates. Nonadherent cells were further depleted of B cells, NK cells, and residual monocytes by negative selection with anti-CD20, anti-MHC class II, and anti-CD56 using goat anti-mouse immunomagnetic Dynabeads M-450 (Dynal Inc., Great Neck, NY). $CD4^+$ and $CD8^+$ T cells were also obtained by negative selection with magnetic beads using anti-CD8 or anti-CD4, respectively. The CD4 and CD8 cells thus obtained were more than 95% pure.

Production and Characterization of Anti-4F9 mAb

The mAb anti-4F9 was produced by standard techniques after immunization of a BALB/c J mouse (The Jackson Laboratories, Bar Harbor, ME) with thymocytes obtained from the new world primate species *Aotus trivirgatus* (13). Hybridoma cultures containing antibodies reactive with human T cells were selected, cloned, and recloned by limiting dilution in the presence of feeder cells. Malignant ascites were then de-

veloped and used for analysis. The hybridoma antibody described here, anti-4F9, was shown to be of the IgG1 subclass by specificity of staining with fluorescein-labeled goat anti-mouse IgG1.

Analysis of Lymphocyte Population with a Fluorescence-Activated Cell Sorter

Single- and two-color fluorescence flow cytometric analyses were performed on an Epics V cell sorter (Coulter Electronics, Hialeah, FL). For single-color analysis, cells were stained with mAb ascites at dilutions of 1/250 to 1/1000, followed by incubation with FITC-conjugated goat anti-mouse $F(ab')_2$. Background fluorescence reactivity was determined using ascites from nonsecreting hybridoma clones. Two-color staining was performed by using anti-4F9–FITC and anti-CD45RA(2H4)–PE or anti-CD45RO(UCHL-1)–biotin followed by streptoavidin–PE.

Immunoprecipitation and Enzymatic Treatment of the 4F9 Ag

Cells were surface labeled with ^{125}I by using the lactoperoxidase method. They were then solubilized in lysis buffer (50 m*M* Tris–HCl, pH 7.4, containing 0.14 *M* NaCl, 1% NP-40, 1 m*M* PMSF, 1 μg/ml pepstatin, 10 m*M* iodoacetamide, 0.1 U/ml aprotinin, 5 m*M* EDTA). The lysate was precleared with Affi-gel–protein A (Bio-Rad, Richmond, CA) plus rabbit anti-mouse Ig (Jackson ImmunoResearch). The precleared lysates were then incubated at 4°C for either 1–2 h or overnight with mAb plus 10 μl of rabbit anti-mouse Ig Ab before precipitation with 100 μl of Affi-gel–protein A. After extensive washing with lysis buffer, immunoprecipitates were eluted by boiling for 5 min in sample buffer (0.1 *M* Tris–HCl, pH 6.8, containing 10% glycerol, v/v, and 1% SDS). The samples were run on a 10% SDS–PAGE under either reducing or nonreducing conditions. Treatment of immunoprecipitated 4F9 Ag with *N*-glycanase, which cleaves N-linked oligosaccharide chains from proteins, was performed as described elsewhere (23). After stopping the reaction by addition of sample buffer, the samples were run on 10% SDS–PAGE.

Cloning and Sequence of cDNA

A cDNA library was constructed from poly(A)$^+$ RNA prepared from PHA/PMA-activated T cells as described (24). The expression cloning was performed by a high-efficiency COS cell expression system as described previously (25). The nucleotide sequence of both strands of the isolated cDNA was determined by using the dideoxy sequencing method (26).

T Cell Proliferation Assays

For preparation of culture plates coated with anti-CD3 alone or anti-CD3 plus other antibodies, 100 μl of protein A-purified anti-CD3 antibody (0.1 μg/ml) was placed in each well of a 96-well flat-bottom plate, which was then incubated for 3 hr at room temperature. After washing three times with PBS, 100 μl of PBS containing the indicated amounts of second antibodies was then plated in each well and incubated for an additional 3 hr at room temperature. Before use, wells were washed three times with PBS. T cells were cultured in triplicate wells at a concentration of 10^5 cells/well in serum-free medium consisting of Iscove's MEM supplemented with 0.1% BSA, 30 μg/ml human transferrin, 10 μg/ml soybean lipids, 4 m*M* L-glutamine, 25 m*M* Hepes

buffer, 0.5% sodium bicarbonate, and 50 μg/ml of gentamicin sulfate. After 4 days in culture, each well was labeled with 1 μCi of [^{3}H]thymidine (ICN Radiochemicals, Irvine, CA) for 18 hr. Cells were then harvested on a PHD cell harvester (Cambridge Technology, Inc, Cambridge, MA), and [^{3}H]thymidine incorporation was measured with a β scintillation counter.

Cell Adhesion Assay

Cell adhesion assays were performed according to the method described previously (27). In brief, 24-well microtiter plates were incubated with 500 μl of mAbs (5 μg/ml) for 3 hr at room temperature, followed by incubation with heat-treated bovine serum albumin (10 min at 80°C) in PBS (5 mg/ml) for 2 hr at room temperature and washing with PBS. T cells were incubated in these plates at 37°C and observed for their attachment and spreading on the substrata under a phase-contrast microscope.

RESULTS

Reactivity of Anti-4F9 with T Cell Subsets

In an attempt to identify novel functional surface molecules on T cells, we have immunized mice with thymocytes obtained from *A. trivirgatus*. This strategy has allowed us to develop a number of mAbs crossreactive with human T cells that have identified molecules having important functions (13, 14). Figure 1A shows a representative staining pattern of anti-4F9 on unfractionated, $CD4^+$, and $CD8^+$ T cells freshly isolated from a healthy donor. The mean percentages reactivity ±SD of anti-4F9 antibody with subsets of T cells from five individuals are as follows: 40.0 ± 9.6 of unfractionated T cells, 51.0 ± 7.5 of $CD4^+$ cells, and 15.2 ± 7.6 of $CD8^+$ cells. Thus, three to four times more CD4 cells than CD8 cells express 4F9. When the expression of 4F9 on the $CD45RO^+$ and $CD45RA^+$ subpopulations in $CD4^+$ cells was compared, the Ag was found to be preferentially distributed on the $CD45RO^+$-$CD45RA^-$ subpopulation. As can be seen in Fig. 1B, the majority (>90%) of $CD4^+$ cells bearing the 4F9 Ag expressed a high density of CD45RO, and two-thirds (65%) of $CD4^+$ cells bearing CD45RO expressed the 4F9 Ag. Conversely, few (<10%) $CD4^+$ cells bearing the 4F9 Ag coexpressed CD45RA, and few (10%) $CD45RA^+CD4^+$ cells were 4F9-positive. These results indicate that the 4F9 Ag is an additional marker for the T cell subset with memory and/or helper/inducer phenotypes ($CD4^+CD45RO^+CD45RA^-$).

Biochemical and Molecular Characterization of the 4F9 Ag

The 4F9 Ag was isolated from surface radioiodinated T cells by immunoprecipitation from cell lysates using the anti-4F9 mAb. Separation of the labeled immunoprecipitates by SDS–PAGE revealed a broad band ranging from approximately 43 to 50 kDa when normal resting T cells and the malignant T cell line H9 were used (Fig. 2A). An identical electrophoretic mobility was observed both under reducing and nonreducing conditions (data not shown), suggesting that this Ag consists of a single chain polypeptide which is not linked by intra- or interchain disulfide bonds. To ascertain the contribution of oligosaccharide side chains to the molecular mass, we performed *N*-glycanase digestion of the immunoprecipitated 4F9 Ag. Extensive digestion with *N*-glycanase resulted in a reduction of the molecular mass to approximately 25 kDa in

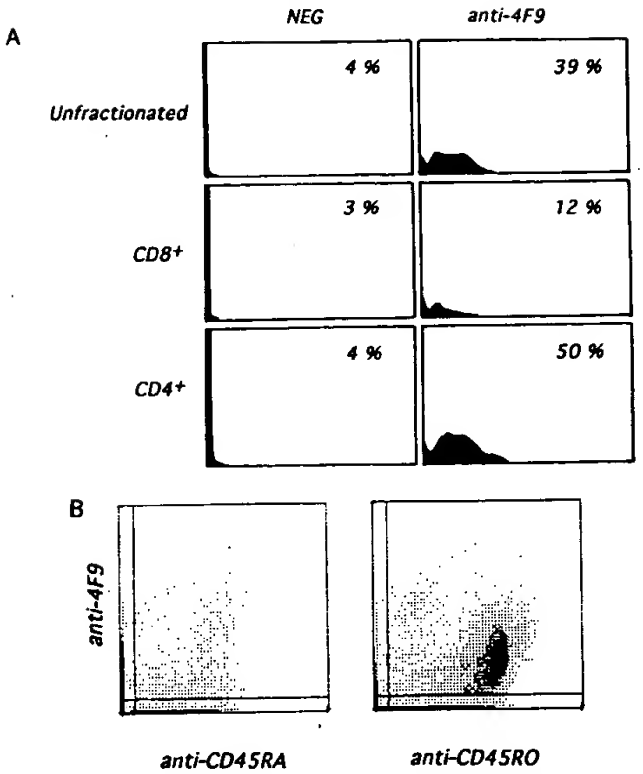

FIG. 1. Cytofluorographic analysis of T cells with anti-4F9. (A) Expression of 4F9 on unfractionated, $CD4^+$, and $CD8^+$ T cells. 4F9 expression was determined by immunofluorescence as described under Materials and Methods and expressed as percentage of cells reactive with anti-4F9. Fluorescence intensity is represented on the horizontal axis on a three-decade lagarithmic scale, and cell number is on the vertical axis. Data are representative of five separate experiments. (B) Relationship between 4F9 expression and CD45RA or CD45RO expression on freshly isolated $CD4^+$ T cells. Two-color analysis was performed as described under Materials and Methods. Data are representative of three separate experiments. In these representative experiments, the actual percentages of cells within each subset were as follows: 31.8% $4F9^+CD45RA^-$, 3.7% $4F9^+CD45RA^+$, 36.8% $4F9^-CD45RA^-$, 27.6% $4F9^-CD45RA^+$, 2.8% $4F9^+CD45RO^-$, 40.0% $4F9^+CD45RO^+$, 35.2% $4F9^-CD45RO^-$, 21.8% $4F9^-CD45RO^+$.

H9 cells (Fig. 2B) as well as peripheral resting T cells (data not shown). In contrast, *O*-glycanase treatment had no effect on the electrophoretic mobility of the 4F9 Ag (data not shown). These results suggest that the 4F9 Ag is expressed at the cell surface as a protein core of 25 kDa which is primarily N-glycosylated resulting in a glycoprotein with M_r of 43–50 kDa.

To further obtain structural information about the 4F9 Ag, its cDNA was isolated from a cDNA library constructed from PHA/PMA-activated T cells using a expression cloning method (5, 24, 25). Analysis of plasmid DNA after three cycles of immunoselection indicated that 8 of 24 clones contained cDNA inserts of 1.6 kb in size. One of these clones was transfected into COS cells by the DEAE-dextran method, and after 72 hr, the expression of 4F9 was examined by indirect immunofluorescence. COS cells transfected with this cDNA, but not those transfected by the pCDM8 vector alone, were found to express 4F9 epitope (data not shown).

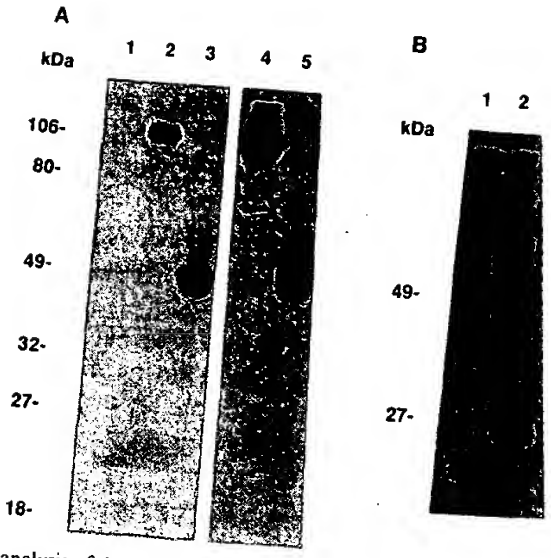

FIG. 2. Biochemical analysis of the molecule recognized by anti-4F9. (A) Peripheral resting T cells (lanes 1–3) and the human T cell line H9 (lane 4 and 5) were surface labeled with ^{125}I and lysed in 1% NP-40 lysis buffer. Precipitates were analyzed on 10% SDS–PAGE under reducing conditions. Lane 1, anti-CD1 (T6) as a negative controle; lanes 2 and 4, anti-CD29 (4B4); lanes 3 and 5, anti-4F9. (B) *N*-Glycanase treatment of 4F9 Ag. Immunoprecipitate obtained from lysates of ^{125}I-labeled H9 cells was digested with *N*-glycanase overnight at 37°C as described under Materials and Methods. After termination of the reaction, the enzyme-treated (lane 2) and the untreated (lane 2) materials were subjected to SDS–PAGE analysis.

Sequence and homology search revealed that the 4F9 Ag is completely identical to a recently identified molecule called R2 (17) (data not shown). R2 cDNA was first isolated by subtractive hybridization from a cDNA library obtained from activated T cells. Moreover, two additional groups (28, 29) have independently reported the isolation of cDNA encoding R2 from cDNA libralies derived from U937 and Molt-4 cell lines using mAbs (anti-IA4 and anti-C33, respectively) as probes. Recently, several membrane proteins having closely related structures have been cloned (17–19). These molecules each have four transmembrane domains which are highly conserved and are proposed to constitute a supergene family (tetra spans transmembrane protein family) (18). Family members include CD9, CD37, CD57, CD63, TAPA-1, CO-029, as well as the *Schistosoma mansoni* antigen Sm23. The highest degree of sequence similarity between the members of this family is observed within the putative transmembrane regions, whereas the amino acid sequences of the predicted extracellular loop diverge from each other. Thus, the 4F9 Ag is a member of this structurally unique transmembrane protein family.

Crosslinking of 4F9 Ag with Anti-4F9 Activates CD4 Cells in Combination with Anti-CD3

The 4F9 Ag is preferentially distributed on the $CD45RO^+CD4$ subset in T cells. Such phenotypic characteristics are reminiscent of several adhesion molecules such as LFA-1, the VLA antigens, CD26, and CD44 (4), all of which, however, are structurally distinct from the 4F9 Ag. One common feature of these adhesion molecules

is their ability to provide costimulatory signals with the CD3/TcR pathway (10, 11, 27, 30, 31). Therefore, we next examined the effect of immobilized anti-4F9 on anti-CD3-dependent CD4 cell proliferation using a previously described T cell culture system (27, 31). In this system, neither immobilized anti-CD3 alone nor anti-4F9 alone could elicit CD4 cell activation. However, when antibodies were used in combination, prominent CD4 cell proliferation was observed in a dose-dependent fashion (Fig. 3). Costimulation induced by anti-4F9 is specific, since crosslinking of anti-CD31 or anti-TQ1 (anti-L-selectin) antibodies with anti-CD3 had no effect in this system.

Immobilized Anti-4F9 Promotes Spreading of T Cells

In addition to the comitogenic effect of immobilized anti-4F9, we noted that T cells cultured on such plates exhibited a prominent change in morphology. To further analyze this phenomenon, we performed adhesion assays using several cell lines expressing 4F9 Ag as described under Materials and Methods. Figure 4 shows representative results when human T cell line H9 cells were used as a target cell. Sixty to eighty percent of H9 cells developed prominent dendritic processes within 60 min after contact with anti-4F9-coated substrata. This spreading of H9 cells was not observed when they were cultured on plates coated with the anti-MHC class I antibody (W6/32) (Fig. 4) or plates not coated with any mAb (data not shown). Since the level of the expression of MHC class I Ag on H9 cells was almost equal to that of 4F9 Ag (data not shown), the spreading induced by anti-4F9 was at least unrelated to Ag density. This anti-4F9-mediated spreading of cells was also observed in PHA-activated T cells and other cell lines expressing the 4F9 Ag. Among the T cell lines we examined, H9 cells exhibited the strongest response in spreading assays. Therefore, additional experiments were performed using H9 cells.

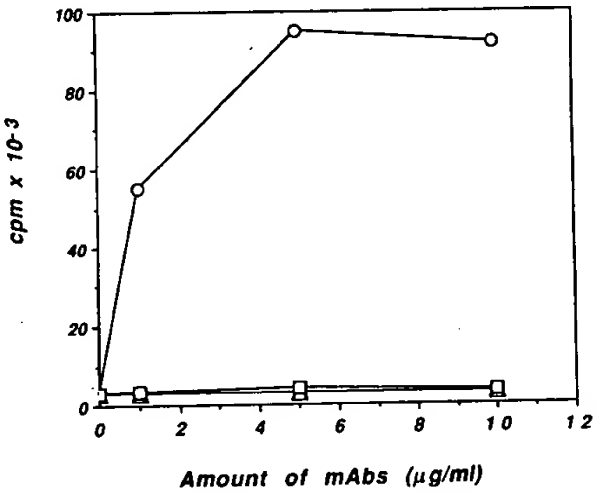

FIG. 3. Comitogenic effect of anti-4F9 on CD3-dependent CD4 cell proliferation. CD4 cells were cultured on plates coated with anti-CD3 (0.1 μg/ml) alone or in combination with anti-4F9 (circles), anti-CD31 (squares), and anti-TQ1 (triangles) antibodies. After 4 days of culture, proliferation was assessed by determining [^{3}H]thymidine incorporation. The data are expressed as the mean of triplicate samples. Each SD was <15%. CD4 cell proliferation cultured on plates coated with each mAb alone was less than 2000 cpm. The data shown are representative of five separate experiments.

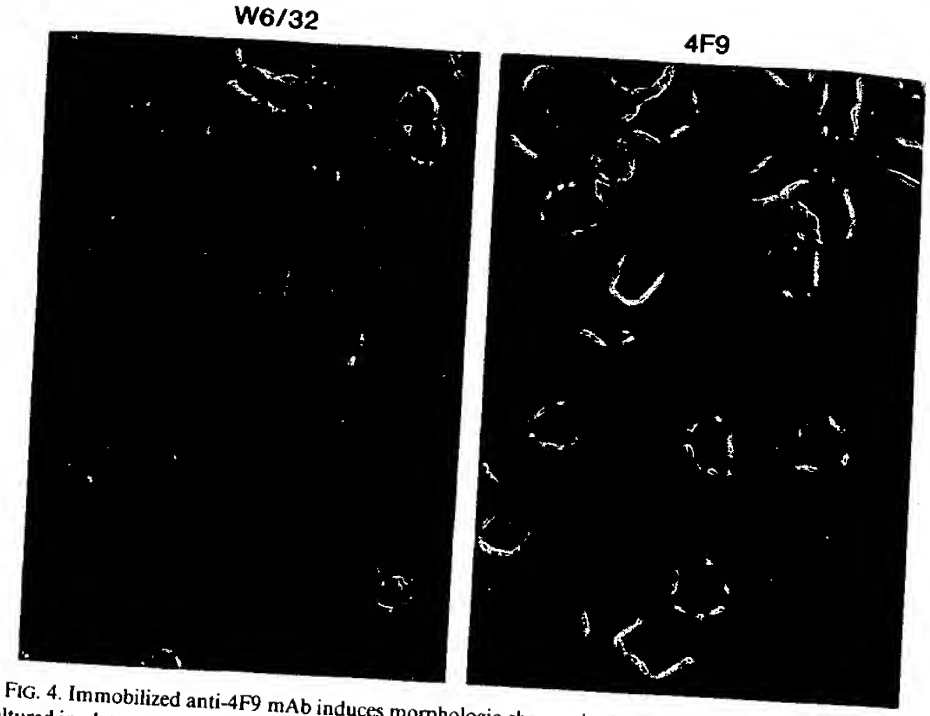

FIG. 4. Immobilized anti-4F9 mAb induces morphologic changes in the human T cell line H9. Cells were cultured in plates coated with anti-4F9 (5 μg/ml) or anti-W6/32 (5 μg/ml). After 60 min, cells were observed for their attachment and spreading on the substrata under a phase-contrast microscope (magnification: 200×).

Cell Spreading Induced by anti-4F9 Is Mediated by Reorganization of Cytoskeleton

Such spreading phenomenon is generally considered to be mediated by a reorganization of cytoplasmic cytoskeleton (32). Therefore, we next investigated whether agents which inhibit cytoskeletal function can block this phenomenon. For this purpose we examined the effect of cytochalasin D and colchicine on anti-4F9-induced spreading of H9 cells. Cytochalasin interferes with microfilament function, and colchicine disrupts microtubules. As shown in Table 1, cell spreading could be completely inhibited by both of these agents. Taken together, our results suggest that the crosslinking of 4F9 Ag on the cell surface by anti-4F9 induced cytoskeletal reorganization, resulting in prominent cell spreading.

TABLE 1

Inhibition of 4F9-Induced H9 Spreading with Cytochalacin D and Colchicine[a]

	10 μM	1 μM	0.1 μM
Cytochalasin D	>90%	>90%	>50%
Colchicine	>90%	>50%	(−)

[a] Cells were cultured in plates coated with anti-4F9 (5 μg/ml) in the presence or absence of indicated reagents.

DISCUSSION

In this report, we described an N-glycosylated 43- to 50-kDa protein defined by anti-4F9, which is primarily expressed on the surface of a subset of human T cells. Molecular cloning and sequencing of a cDNA encoding this molecule revealed that the Ag is totally identical to R2, a molecule cloned by subtractive hybridization. The 4F9/R2 has a unique structure containing four putative transmembrane domains which is a common feature of a newly identified gene family (18) of membrane proteins that includes CD9, CD37, CD53, CD64, TAPA-1, CO-029, and Sm23. The highest degree of sequence homology between the members of this family is observed within their transmembrane regions (18), suggesting that these regions may be critical for the structural integrity of these proteins and their interactions with other cellular elements. In contrast, the hydrophilic extracellular region exhibits diversity presumably because of its involvement in binding different ligands. Despite its defined structure, the functions of members of this protein family are largely unknown (18). By analyzing effects of anti-4F9 mAb on T cell function, we demonstrated that the 4F9 Ag may act as a costimulatory molecule for T cell proliferation. Furthermore, crosslinking of the 4F9 Ag with its mAb immobilized on plastic plates induced prominent spreading involving in cytoskeletal reorganization. These results suggest that the 4F9 Ag may play a role in T cell activation and adhesion. The distribution of this molecule on the $CD4^+CD45RO^+$ subset of T cells may contribute to the distinct activation profile and functional repertoire of these cells.

Several studies have shown that some of the members of the tetra spans transmembrane protein family are involved in the regulation of cell proliferation (19, 34). For example, mAbs against CD37 inhibited the activation of B cells induced by anti-CD20 mAb and B-cell growth factor but enhanced the mitogenic effect of anti-Ig antibodies on the same cells (34). Similary, antibodies to TAPA-1 exert an antiproliferative effect on a variety of hematopoietic cell lines in culture (19). The costimulatory effect of immobilized anti-4F9 on CD3-dependent T cell proliferation further supports the idea that the 4F9 Ag may play a role in cell growth. As described previously, immobilized anti-CD3 fails to drive extensively monocyte-depleted T cells to proliferate (27, 31). Like the physiologic Ag-specific response, T cells require additional costimuli. Several cell-surface structures have been shown to be able to provide costimulation by coimmobilizing either their natural ligands or specific antibodies with anti-CD3 on plastic plates (10, 27, 30, 31, 33). Most of these costimulatory molecules function as adhesion receptors which mediate contact of T cells with other cell types or ECM proteins. This may suggest that ligands or antibodies immobilized on substrata may facilitate the response to anti-CD3 in part by augmenting adhesion, and thereby increasing the surface area of the cell that is in contact with anti-CD3. However, enhanced adhesion alone appears unlikely to explain the costimulatory effect. First, raising the concentration of anti-CD3 on plastic does not induce proliferation of highly purified T cells (27). Second, antibodies or ligands (counter-receptors) against certain other adhesion structures do not result in costimulation (our present data and 30). Finally, a number of studies have demonstrated that ligation of costimulatory molecules with their specific ligands or antibodies directly transmits signals into the T cell (35, 36). Treatment of a hematopoietic cell line U937 with anti-IA4, which recognizes an identical molecule to the 4F9 Ag, was shown to induce increased intracellular Ca^{2+} concentration (28). We also observed that crosslinking the 4F9 Ag with its mAb enhanced Ca^{2+} influx

dependent on CD3 ligation (unpublished data). Taken together, the data suggest that the costimulation induced by the anti-4F9 is not a simple reflection of increased adherence or stabilization of the T cells to submitogenic anti-CD3.

We showed that crosslinking 4F9 Ag induces cytoskeletal reorganization in a variety of T cells as demonstrated by prominent cell spreading. It has been established that the interaction between the cell-surface binding site and adhesion ligands triggers an active cytoskeletal response at the site of initial adhesion, resulting in production of cell extensions that are involved in cell spreading (32, 37). Antibodies against cell surface adhesion structures have been shown to mimic this process when they are immobilized on substrata (38, 39). The mechanism triggering this phenomenon is unclear, although direct physical interaction with cytoskeletal elements has been postulated in the case of integrins (37, 40). Recent studies have shown that selective reorientation of cytoskeletal proteins and intracytoplasmic organelles including Golgi apparatus and the microtubule organizing center could be observed in both CTL and T helper cells upon interaction with Ag-bearing cells (41). In addition to Ag-specific TCR, other Ag-nonspecific adhesion molecules such as LFA-1 were shown to be involved in this process (41). These cytoskeletal changes are assumed to play a role in T cell effector functions such as the polar secretion of cytolytic granules or lymphokines. Our findings suggest that the 4F9 Ag may be also involved in signaling cytoskeletal reorganization in T cells.

Of special interest is a report by Fukudome *et al.* (33) who demonstrated that antibodies agaist C33 Ag (shown to be identical to the 4F9 Ag) could inhibit syncytium formation resulting from HTLV-1-induced cell fusion. Syncytium formation requires specific cell-to-cell contact mediated by interaction between viral glycoproteins expressed on HTLV-1-infected cells and an undefined receptor on target cells. In the case of HIV-induced syncytium formation, anti-LFA-1 antibody, as well as anti-CD4, have been shown to block this process (42). Given our present data, the 4F9/C33 Ag may be involved in the process of HTLV-1-induced syncytium formation by promoting cell adhesion. It is also interesting that the CD9 Ag expressed by human platelets, another member of tetra spans transmembrane protein family, is noncovalently associated with the major adhesion molecule in the platelets, integrin GPIIb/IIIa (43). Some of mAbs against CD9 activate platelet aggregation mediated via interaction between fibrinogen and fibrinogen receptor (GPIIb/IIIa). This suggests that CD9 is a functional and physical component of the integrin/adhesion receptor complex (43). Furthermore, mAbs reactive with TAPA-1 have been shown to induce homotypic lymphoid cell aggregation (19). Taken together, members of this novel protein family may be commonly involved in cell adhesion. Thus far, however, we have been unable to demonstrate any inhibition of T cell attachment to other cells or extracellular matrix proteins by anti-4F9 (data not shown). Further studies will be necessary to demonstrate whether or not the 4F9 Ag is really an adhesive structure or a regulatory molecule in the cell adhesion process.

In summary, using a mAb anti-4F9 reactive with a T cell surface protein, we have characterized the functional, biochemical, and molecular nature of this molecule. Based on their unique structures, members of the tetra spans transmembrane protein family are postulated to function as ion channels or transportors. In indirect ways via such mechanisms, they may play a role in cell growth and adhesion. Future studies will be necessary to determine their ligands, associated molecules, and signal transduction pathways. Specific
studies defining the biologi

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duction pathways. Specific antibodies and cDNA will be important tools for these studies defining the biological role of this relatively new and unique supergene family.

ACKNOWLEDGMENTS

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Class I–restricted Cross-Presentation of Exogenous Self-Antigens Leads to Deletion of Autoreactive CD8+ T Cells

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Summary

In this report, we show that cross-presentation of self-antigens can lead to the peripheral deletion of autoreactive $CD8^+$ T cells. We had previously shown that transfer of ovalbumin (OVA)-specific $CD8^+$ T cells (OT-I cells) into rat insulin promoter–membrane-bound form of OVA transgenic mice, which express the model autoantigen OVA in the proximal tubular cells of the kidneys, the β cells of the pancreas, the thymus, and the testis of male mice, led to the activation of OT-I cells in the draining lymph nodes. This was due to class I–restricted cross-presentation of exogenous OVA on a bone marrow–derived antigen presenting cell (APC) population. Here, we show that adoptively transferred or thymically derived OT-I cells activated by cross-presentation are deleted from the peripheral pool of recirculating lymphocytes. Such deletion only required antigen recognition on a bone marrow–derived population, suggesting that cells of the professional APC class may be tolerogenic under these circumstances. Our results provide a mechanism by which the immune system can induce $CD8^+$ T cell tolerance to autoantigens that are expressed outside the recirculation pathway of naive T cells.

Several mechanisms play a role in tolerance induction to extra thymic self-antigens. For class I–restricted $CD8^+$ T cells, ignorance, anergy, and deletion can operate to render an animal tolerant to antigen expressed in peripheral tissues (1–14). However, the current dogma provides an interesting dilemma with regard to our understanding of how tolerance is achieved. Anergy and deletion both require interaction of T cells with the self-antigen, and naive T cells are thought not to recirculate through nonlymphoid tissue (15). Thus, those $CD8^+$ T cells specific for antigens expressed only in nonlymphoid tissues, e.g., in the β cells of the pancreas, should not be susceptible to these forms of tolerance. This leaves ignorance as the only mechanism for avoiding autoimmunity to such antigens, a somewhat unsatisfactory situation because activated $CD8^+$ T cells have wider recirculation pathways than naive cells (15), and can potentially cause autoimmune damage in tissues previously ignored (3, 4, 10). Consequently, we might expect autoimmunity to be more prevalent, or that additional tolerogenic mechanisms exist.

To date, many studies examining peripheral tolerance of $CD8^+$ T cells have used MHC molecules as their model autoantigens (5–10, 14, 16). These molecules are seen only in an unprocessed form, and therefore only on those cells that themselves express the autoantigen. Although such studies have contributed to our understanding of the fate of autoreactive $CD8^+$ T cells, they have not allowed for the possible effects of processing and presentation of tissue antigens by professional APCs.

Peptide antigens presented by MHC class I molecules are generally thought to be derived from intracellularly synthesized proteins (17–19). However, exogenous antigens can also be presented by class I MHC molecules under certain circumstances (20–26) and the induction of CTL via this exogenous pathway for class I–restricted presentation has been referred to as cross-priming. We have recently shown that such presentation, when applied to self-antigens, can lead to the activation of autoreactive $CD8^+$ T cells (27). These studies used the rat insulin promoter (RIP)[1]–mOVA transgenic mouse model, where a membrane-bound form of ovalbumin (mOVA) was expressed by pancreatic β cells, kidney proximal tubular cells, the thymus, and in the testis of male mice. Transgenic OVA-specific $CD8^+$ T cells (OT-I cells) adoptively transferred into RIP–mOVA mice were activated in the lymph nodes draining OVA-expressing tissues. This activation was due to class I–restricted presentation of exogenously derived OVA on a bone marrow–derived APC population. Here, we investigate the fate of autoreac-

[1] *Abbreviations used in this paper:* HA, hemagglutinin; mOVA, membrane-bound form of ovalbumin; OT-I, OVA-specific $CD8^+$ T cells; RIP, rat insulin promoter; TG, thymus grafted.

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tive CD8+ T cells activated by this cross-presentation pathway and provide evidence that these cells are deleted.

Materials and Methods

Mice. All mice were bred and maintained at the Walter and Eliza Hall Institute of Medical Research. For all experiments, female mice between 8 and 16 wk of age were used. RIP-mOVA and OT-I transgenic mice were generated and screened as previously described (27, 28).

Adult Thymectomized, Thymus-grafted, Bone Marrow Chimeras. Adult thymectomized, thymus-grafted, bone marrow chimeric RIP-mOVA mice (TG-RIP mice) and nontransgenic mice (TG-nontransgenic mice) were generated as described (9, 14). In brief, 2–6 wk after adult thymectomy RIP-mOVA mice and their nontransgenic littermates were lethally irradiated with 900 cGy and reconstituted with T cell–depleted bone marrow from OT-I mice. The next day, radioresistant T cells were depleted with 100 μl of T24 (anti-Thy-1) ascites intraperitoneally. 1–2 wk after irradiation, mice were grafted with a sex-matched 1,500 cGy irradiated thymus graft from a nontransgenic newborn B6 mouse under the kidney capsule. This approach ensured that OT-I cells were not deleted intrathymically due to aberrant expression of OVA in this tissue.

Preparation of OT-I Cells for Adoptive Transfer. OT-I RAG-1$^{-/-}$ cells were prepared from LN and spleens of transgenic mice as described (27). In brief, erythrocytes and macrophages were removed by treatment with the anti-heat stable antigen mAb J11d and complement. OT-I cells from RAG-1$^{-/-}$ mice were of a naive phenotype (CD44^{lo}, CD69^{-}, IL-2R^{-}). 0.25–6 × 10^6 cells were injected intravenously into recipient mice.

5,6-Carboxy-Succinimidyl-Fluoresceine-Ester Labeling of OT-I Cells. 5,6-carboxy-succinimidyl-fluoresceine-ester (CFSE) labeling was performed as previously described (29–31). OT-I cells were resuspended in PBS containing 0.1% BSA (Sigma Chem. Co., St. Louis, MO) at 10 × 10^6 cells/ml. For fluorescence labeling, 2 μl of a CFSE (Molecular Probes, Eugene, Oregon) stock solution (5 mM in DMSO) were incubated with 10 × 10^6 cells for 10 min at 37°C.

FACS® Analysis. LN or spleen cells were stained for three-color FACS® analysis as described (10), using the following mAbs: PE-conjugated anti-CD8 (YTS 169.4) was from Caltag (San Francisco, CA). Biotinylated anti-CD69 (H1.SF3) and PE-labeled anti-L-selectin (Mel-14) were from PharMingen (San Diego, CA). Anti-Vα2 TCR (B20.1) and anti-Vβ5.1/2 TCR (MR9-4) mAbs (27) were conjugated to biotin or to FITC. Biotin-labeled Abs were detected with Streptavidin (SAVP)-Tricolor (Caltag). Analysis was performed on a FACScan® using Lysis II software. Live gates were set on lymphocytes by forward and side scatter profiles. 10,000–20,000 live cells were collected for analysis. OT-I donor cells in the LNs from recipient mice were identified by staining for Vα2$^+$ Vβ5$^+$ CD8$^+$ cells.

For analysis of fluorescent labeled cells, 50,000 CD8$^+$ cells were collected and analyzed using WEASEL software (F. Battye, Walter and Eliza Hall Institute, Melbourne, Australia).

Histology. Tissues were fixed in Bouin's solution and paraffin sections were stained with hematoxylin and eosin using standard methods.

Results

Activation of OVA-specific CD8$^+$ T Cells by Cross-presentation in RIP-mOVA Mice. RIP-mOVA mice express a membrane-bound form of OVA in the β cells of the pancreas, the proximal tubular cells of the kidney, the thymus, and in the testis of male mice (27). When OVA-specific CD8$^+$ T cells from the OT-I transgenic line (OT-I cells) were transferred into RIP-mOVA mice, they were activated in the draining lymph nodes of OVA-expressing tissues (27). In this report, we have used a novel and more sensitive method for the identification of proliferating cells in vivo (29–31). OT-I cells were labeled with the fluorescent dye CFSE and then transferred into RIP-mOVA mice. When CFSE-labeled cells divide, the two daughter cells receive approximately half of the original fluorescence, and their progeny a quarter, and so on. Thus, a cell that has divided n times will exhibit a 2^n-fold reduced fluorescence intensity. Therefore, on a FACS® histogram, separate peaks appear for cells that have divided 1–8 times. After nine cell cycles, the fluorescent dye is diluted to background intensity. Fig. 1 shows the CFSE profiles of 5 × 10^6 OT-I cells transferred into RIP-mOVA mice 43 h earlier. Multiple peaks are seen only in the renal (Fig. 1 *A*) and pancreatic (Fig. 1 *B*) lymph nodes, confirming that OT-I cells were activated and proliferated only in these sites.

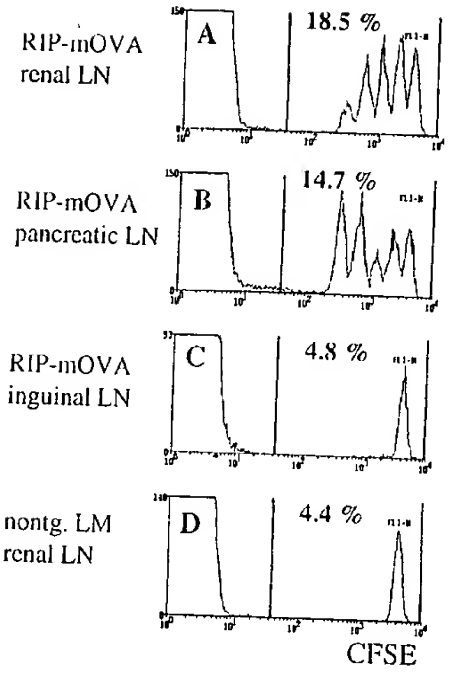

Figure 1. Proliferation of OT-I cells in lymph nodes draining OVA-expressing tissues of RIP-mOVA mice. 5 × 10^6 CFSE-labeled OT-I cells were injected intravenously into RIP-mOVA mice and nontransgenic littermates. 43 h later, lymphocytes prepared from the renal (*A*), pancreatic (*B*), and inguinal (*C*) LN of RIP-mOVA mice, and from the renal LN of nontransgenic recipients were analyzed by flow cytometry. Profiles were gated on CD8$^+$ cells.

Using the above technique, divided cells were first apparent at 25 h after transfer (data not shown). After 43 h, some OT-I cells had divided four times (Fig. 1), six times within 52 h (data not shown), greater than eight times

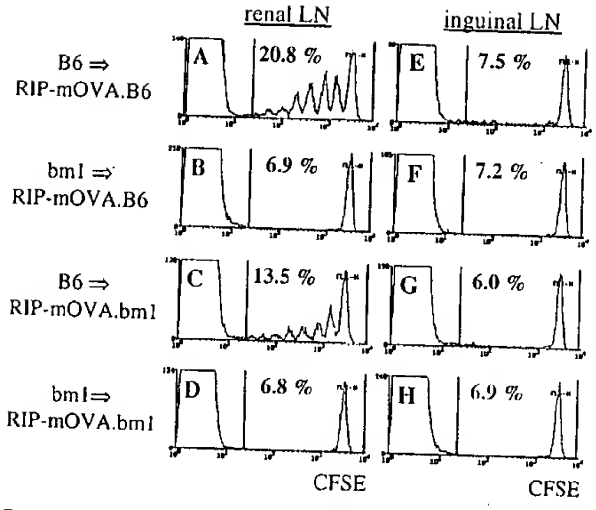

Figure 2. Bone marrow-derived APC activate OT-I cells in the draining LN of RIP-mOVA mice. 5 × 10^6 CFSE labeled OT-I cells were transferred into RIP-mOVA mice backcrossed to B6 (*A, B, E, F*) or bm1 (*C, D, G, H*) and grafted with B6 (*A, C, E, G*) or bm1 (*B, D, F, H*) bone marrow. After 3 d, renal (*A–D*) and inguinal (*E–H*) lymph node cells were examined by flow cytometry. Profiles were gated for $CD8^+$ cells.

within 68 h (Fig. 2). Therefore, one cell cycle required ~4.5 h in vivo.

These results indicate that OT-I cells were able to respond to antigen presented in the lymph nodes that drained OVA-expressing tissues. Previously, we showed that in the absence of a bone marrow-derived APC capable of presenting OVA to OT-I cells, no proliferation was observed (27). To determine whether OVA presentation by such bone marrow-derived APCs alone was sufficient to induce OT-I cell proliferation, we took advantage of bm1 mice, which express a mutation in the K^b molecule such that K^{bm1} cannot present OVA to OT-I cells (32). RIP-mOVA mice, which were crossed onto the bm1 haplotype (RIP-mOVA.bm1), were lethally irradiated and reconstituted with B6 bone marrow (B6→RIP-mOVA.bm1). In these chimeric mice, K^b is expressed by bone marrow-derived cells but not by peripheral tissue cells such as islet β cells or kidney proximal tubular cells. 5 × 10^6 CFSE-labeled OT-I cells were adoptively transferred into the chimeric RIP-mOVA mice and, 3 d later, lymphoid tissues were analyzed by flow cytometry (Fig. 2). Proliferation of OT-I cells was observed in the renal (Fig. 2 *C*) and pancreatic (data not shown) nodes, but not in the inguinal lymph nodes of B6→RIP-mOVA.bm1 chimeras (Fig. 2 *G*). This result showed that antigen presentation by bone marrow-derived cells was sufficient to induce proliferation of OT-I cells. The proliferation was not as intense as in normal RIP-mOVA mice (see Fig. 1), but was comparable to that seen in B6→RIP-mOVA.B6 control chimeras that were entirely of the B6 haplotype (Fig. 2, *A* and *E*). This implied that the less vigorous response seen in chimeric mice may be the result of irradiation. As previously reported (27), OT-I cells were not activated when the bone marrow compartment was of the bm1 haplotype (bm1→RIP-mOVA.B6 or bm1→mOVA.bm1), indicating that antigen presentation by a bone marrow-derived cell was not only sufficient, but also essential for OT-I activation.

Deletion of Adoptively Transferred OT-I Cells in the Periphery of RIP-mOVA Mice. The above results indicate that a bone marrow-derived APC was capable of processing and presenting antigens expressed by peripheral tissues for activation of autoreactive $CD8^+$ T cells. To determine how the immune system normally copes with such autoreactive cells, we examined the ultimate fate of these cells. To detect adoptively transferred OT-I cells in unirradiated recipients several weeks after transfer, it was necessary to inject at least 5 × 10^6 cells. However, under these circumstances OT-I cells infiltrated the pancreatic islets after day 3, and caused diabetes in 100% of 16 RIP-mOVA mice by day 9 (data not shown). Smaller numbers of cells, e.g., 0.25 × 10^6 cells, did not cause diabetes in 25 recipients, but detection of these few cells was not possible several weeks after transfer, even in nontransgenic controls. Presumably, OT-I cells were activated after transfer in both cases, but only the larger dose caused sufficient destruction to result in diabetes. To avoid the problem of β cell destruction, we transferred 6 × 10^6 OT-I cells into B6→RIP-mOVA.bm1 chimeric mice, in which OT-I cells could recognize antigen on the cross-presenting bone marrow-derived APCs, but could not interact with OVA-expressing peripheral tissues of the bm1 haplotype. After 8 wk, far fewer OT-I cells

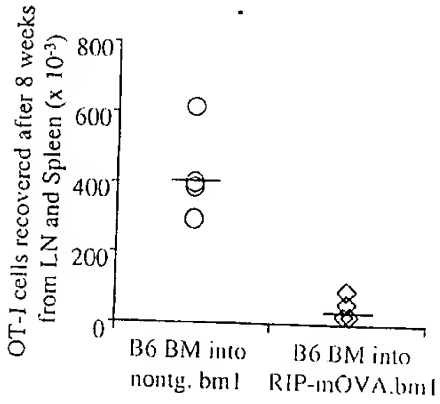

Figure 3. OT-I cells are deleted in response to recognition of antigen on cross-presenting APC. Bone marrow from B6 mice was grafted into RIP-mOVA.bm1 mice and nontransgenic littermates. 14 wk later, 6 × 10^6 OT-I cells were adoptively transferred, and after 8 wk the number of $V\alpha2^+V\beta5^-CD8^+$ cells in the LN and spleen of the recipients was determined by flow cytometry. The proportion of $V\alpha2^+V\beta5^+$ cells in the $CD8^+$ population was 7.5–10% in nontransgenic, and 1.4–3.5% in transgenic recipients. An average of 1.4% of $CD8^+$ cells were $V\alpha2^+V\beta5^+$ in uninjected mice. To derive the total number of OT-I cells, this 1.4% was subtracted from the proportion of $V\alpha2^+V\beta5^+$ cells in the $CD8^+$ cells in experimental mice and the difference was multiplied with the proportion of $CD8^+$ T cells in the live cells and with the number of live cells. These results are representative for two such experiments consisting of four mice per group.

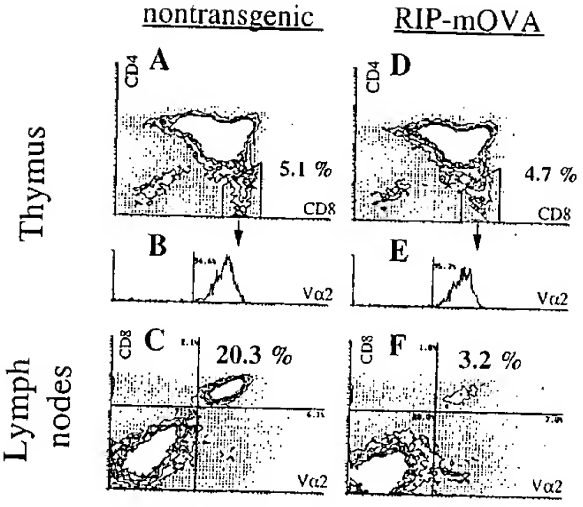

Figure 4. Peripheral deletion of OT-I cells that mature in the thymus of TG-RIP mice. Thymus grafts from TG-littermate mice (*A* and *B*) and TG-RIP mice (*D* and *E*) were analyzed for $CD4^+$, $CD8^+$, and $V\alpha2^+$ cells by flow cytometry 16 wk after bone marrow reconstitution. Expression of Vα2 by $CD4^-CD8^+$ cells is shown in the histograms *B* and *E*. LN cells of the same transgenic (*C*) and nontransgenic (*F*) mice were stained for $CD8^+$ and $V\alpha2^+$ expression. The same staining conditions for Vα2 were used for thymus and LN cells. The data shown here is representative for eight pairs of manipulated mice investigated.

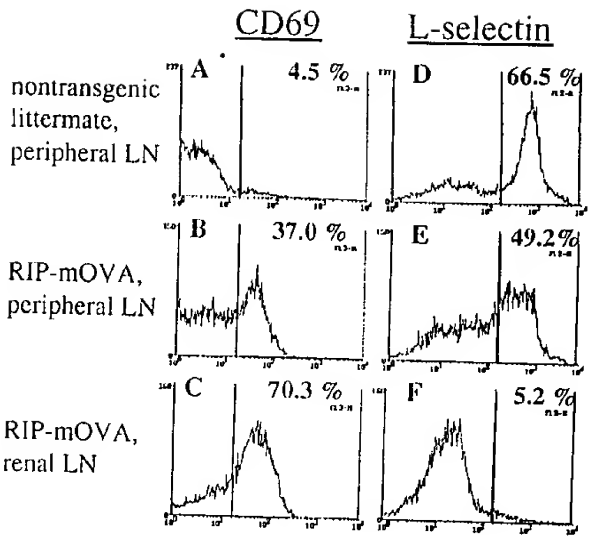

Figure 5. Analysis of the activation status of OT-I cells in TG-RIP mice. Expression of CD69 (*A–C*) and L-selectin (*D–F*) by $V\alpha2^+$ lymphocytes from the peripheral LN of TG-nontransgenic mice (*A* and *D*) and the peripheral (*B* and *E*) and renal (*C* and *F*) LN of TG-RIP mice.

were recovered from the lymphatic tissues of B6→RIP-mOVA.bm1 mice than from nontransgenic B6→bm1 mice (Fig. 3). These data suggest that OT-I cells were deleted after recognizing exogenously processed OVA on bone marrow–derived APC in the draining lymph nodes of OVA-expressing tissue.

Deletion of Constitutively Produced OT-I Cells in the Periphery of RIP–mOVA Mice. The adoptive transfer of 5×10^6 OT-I cells contrasts with the normal situation where small numbers of newly matured cells enter the periphery from the thymus each day. We reasoned that diabetes may have occurred because the normal tolerogenic mechanisms were unable to cope with such a large number of injected T cells.

To create a more physiological situation where OVA-specific $CD8^+$ T cells would be generated continuously in the thymus, RIP–mOVA mice were manipulated to ensure that OVA could not be expressed in this compartment. This was achieved by thymectomizing RIP–mOVA mice and then grafting them with a nontransgenic B6 thymus. Such mice were then lethally irradiated and reconstituted with bone marrow from OT-I mice, and designated thymus-grafted RIP–mOVA mice (TG-RIP mice). This approach has been successfully used to exclude the effect of aberrant thymic antigen expression in other models (10, 14).

In contrast with the RIP–mOVA mice given large numbers of OT-I cells, which became diabetic within 9 d, only 1 of 12 TG-RIP mice developed the disease when followed for >116 d. Analysis of the thymus grafts 4 mo after implantation showed that OT-I cells ($CD8^+CD4^-V\alpha2^+$ cells) were able to mature in TG-RIP mice (Fig. 4). The proportion of mature OT-I cells in the thymus was equivalent to that of nontransgenic controls (Fig. 4, *A* and *D*), supporting the view that the thymic deletion reported earlier for the double-transgenic mice (27) was the result of aberrant thymic expression of mOVA.

Because OT-I cells matured in the thymus of TG-RIP mice, we could examine their fate after release into the peripheral immune system. Flow cytometric analysis of the lymph node populations of TG-RIP mice showed a significant reduction in the proportion of these cells relative to that seen in TG-nontransgenic mice ($4.9 \pm 2.4\%$ versus $25.1 \pm 8.2\%$, $n = 8$) (Fig. 4, *C* and *F*). Calculation of the total number of OT-I cells in the spleen and lymph node populations indicated that there was approximately a five-fold reduction of these cells in TG-RIP mice ($2.28 \pm 1.21 \times 10^6$ versus $12.06 \pm 2.10 \times 10^6$; $n = 8$). The remaining OT-I cells in TG-RIP mice proliferated in vivo after restimulation with antigen, demonstrating that they were not anergic (data not shown). These data strongly suggest that OVA-specific $CD8^+$ T cells were lost and probably deleted once they entered the periphery.

Consistent with an active deletional process occurring in these mice, OT-I cells from the peripheral lymphatic tissues of TG-RIP mice expressed elevated levels of the activation marker CD69 (Fig. 5, *A–C*) and decreased levels of L-selectin (Fig. 5, *D–F*) relative to that seen in TG-nontransgenic control mice. The proportion of activated OT-I cells was even higher in the lymph nodes draining OVA-expressing tissues (Fig. 5, *C* and *F*), suggesting that activation of OT-I cells in TG-RIP mice also occurred in these draining lymph nodes, presumably by the same cross-presentation mechanism that activates adoptively transferred OT-I cells.

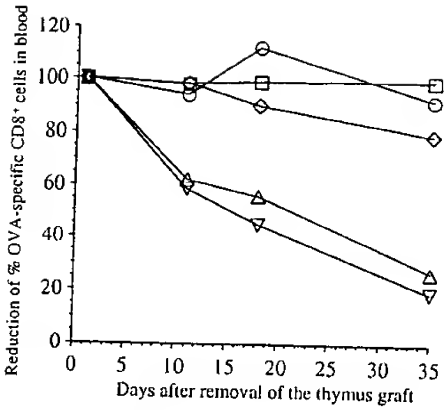

Figure 6. Disappearance of OT-I cells in TG-RIP mice after removal of their thymus graft. Thymus graft was removed from TG-RIP mice and TG-nontransgenic controls 3 mo after implantation. The proportion of $CD8^+$ peripheral blood cells that were $V\alpha2^+V\beta5^-$ was then determined by flow cytometry on days 2, 11, 18, and 35 after thymectomy. The proportion found at day 2 after removal of the thymus grafts was considered 100% in this particular mouse, and the following values were given as a percent of this starting value. TG-RIP, not operated (○); TG-RIP, thymus graft removed (▽); TG-RIP, thymus graft and left kidney removed (△); TG-nontransgenic littermate, not operated (□); TG-nontransgenic littermate, thymus graft removed (◇).

To determine the fate of those few activated OT-I cells remaining in the periphery of TG-RIP mice, the thymus grafts were removed 3 mo after implantation to stop further T cell production. The proportion of OT-I cells was then examined in the blood at various later timepoints. This revealed a continuous decline in the proportion of OT-I cells in TG-RIP mice (Fig. 6), consistent with the idea that a continuous deletion process was in operation. These few remaining cells were also able to proliferate upon restimulation with antigen in vivo (data not shown).

Discussion

There are now numerous reports showing that cross-presentation of exogenous antigen can prime class I–restricted CTL responses (33). It has also been shown to induce tolerance in the thymus (23). Here, we show that cross-presentation can induce peripheral tolerance that appears to operate via a deletional process.

Although our data strongly suggest that OT-I cells were deleted in TG-RIP mice, an alternative possibility is that these cells had relocated to extralymphoid sites. However, because few OT-I cells were seen in nonlymphatic tissues, apart from small numbers of cells in the pancreatic islets (data not shown), this possibility seems remote. It should be emphasized that the mild islet infiltration observed is unlikely to account for the loss of $\sim10^7$ cells from the secondary lymphoid organs of the TG-RIP mice.

Deletion has been reported as a likely mechanism of extrathymic tolerance for several introduced antigens, including superantigens (34, 35), viruses (36), soluble peptides (37, 38), and protein (39), and for some self-antigens in transgenic models (8, 14, 16, 40). The general belief is that this form of tolerance results from exhaustive differentiation (34). T cells are stimulated so extensively by antigen that they proceed to end-stage effectors with limited lifespan. Such a mechanism is consistent with the observed activation and proliferation that precedes deletion in the RIP–mOVA model.

Our findings provide a pathway whereby $CD8^+$ T cells can be tolerized to self-antigens expressed in tissues outside the normal recirculation pathway for naive T cells. As long as the antigen can gain access to the exogenous cross-presentation pathway, host $CD8^+$ T cells should be stimulated to die eventually. Thus, as newly derived autoreactive $CD8^+$ T cells enter the peripheral pool, they will encounter their autoantigen on the cross-presenting APC in lymph nodes that drain the appropriate tissues. As a result, activation will follow and lead to deletion, thus limiting the accumulation of ignorant autoreactive cells in secondary lymphoid compartments. This model is at odds with the previously reported induction of diabetes in virus-primed transgenic mice expressing viral antigens in the islet β cells (3, 4), which suggests that ignorant naive $CD8^+$ T cells remained in the peripheral circulation. However, the type and concentration of the antigen and the affinity of the TCR in the responding T cell population are likely to affect the efficacy of cross-presentation leading to deletion. In support of this conclusion, we have preliminary evidence using newly generated transgenic lines that the level of antigen expressed affects the extent of cross-presentation (our unpublished observations). In addition, data obtained using transgenic mice expressing hemagglutinin (HA) under the control of the rat insulin promoter (RIP–HA mice), support the idea that antigens expressed in the islets are not always ignored but can activate $CD8^+$ T cells leading to tolerance induction. RIP–HA mice crossed to TCR transgenic mice, which produced large numbers of class I–restricted HA-specific T cells, became diabetic (41), indicating that HA-specific $CD8^+$ T cells entered the periphery of RIP–HA mice, where they were activated by islet antigens, perhaps by cross-presentation. Despite this observation, RIP–HA single-transgenic mice showed HA-specific CTL tolerance (11), suggesting that the activation process seen in double-transgenic mice may lead to tolerance induction when the precursor frequency is low, as in a normal T cell repertoire.

It is not clear why this form of priming should lead to loss of activated cells when most other described cases of cross-priming result in immunization. Perhaps it relates to the continuous presence of the priming antigen, which provides repeated stimuli to the responding population to the point of exhaustion. Alternatively, because there is specific tolerance to OVA in the $CD4^+$ T cell compartment in our model (our unpublished observations), deletion of $CD8^+$ cells may relate to a lack of $CD4^+$ T cell help, which appears to be necessary for the induction of some $CD8^+$ T cell responses (42–44). Thus, when $CD8^+$ T cells were confronted with antigen in the absence of $CD4^+$ T cells, only a transient response followed after which all the antigen-specific T cells died (45).

Deletion of OT-I cells in TG-RIP mice is consistent with a model in which newly matured OT-I cells enter the periphery of RIP–mOVA mice, recirculate until they come into contact with antigen in the draining lymph nodes of OVA-expressing tissues, and are then activated and deleted. Such activation-induced deletion could occur in one of two ways: either directly as a result of activation on the bone marrow–derived cross-presenting APC, or indirectly because only activated OT-I cells are able to recirculate through nonlymphoid tissues where they can encounter OVA-expressing tissues and there be deleted. The observed deletion of OT-I cells adoptively transferred into B6→RIP-mOVA.bm1 mice indicates that secondary encounter with antigen on peripheral tissues is not essential for deletion to occur. However, it is important to state that although presentation of OVA on the bone marrow–derived compartment was sufficient to lead to deletion, the additional ability to encounter antigen on peripheral tissue may enhance deletion. This will be examined in future studies.

Our results provide evidence for an extrathymic mechanism capable of inducing the loss of $CD8^+$ T cell responding to self-antigens expressed in tissues outside the lymphoid compartment. Because such tissues are normally not directly accessible to naive $CD8^+$ T cells, the absence of this deletion mechanism would allow accumulation of naive autoreactive $CD8^+$ T cells. These could be primed to effector CTL with wider recirculation pathways after a strong environmental stimulus, thus leading to autoimmunity. We speculate that the continual activation and deletion of small numbers of autoreactive $CD8^+$ T cells by cross-presentation will not result in significant autoimmune damage.

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Peripheral tolerance through clonal deletion of mature CD4⁻CD8⁺ T cells

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Abstract

Transgenic mice bearing the αβ transgenes encoding a defined T cell receptor specific for the male (H-Y) antigen presented by the H-2D^b class I MHC molecule were used to study mechanisms of peripheral tolerance. Female transgenic mice produce large numbers of functionally homogeneous CD8$^+$ male antigen-reactive T cells in the thymus that subsequently accumulate in the peripheral lymphoid organs. We have used three experimental approaches to show that male reactive CD8$^+$ T cells can be eliminated from peripheral lymphoid organs after exposure to male antigen. (i) In female transgenic mice that were neonatally tolerized with male spleen cells, male reactive CD8$^+$ T cells continued to be produced in large numbers in the thymus but were virtually absent in the lymph nodes. (ii) Injection of thymocytes from female transgenic mice into female mice neonatally tolerized with the male antigen, or into normal male mice, led to the specific elimination of male-reactive CD8$^+$ T cells in the lymph nodes. (iii) Four days after male lymphoid cells were injected intravenously into female transgenic mice, male antigen-reactive CD8$^+$ T cells recovered from the lymph nodes of recipient mice were highly apoptotic when compared to CD4$^+$ (non-male reactive) T cells. These data indicate that tolerance to extrathymic antigen can be achieved through elimination of mature T cells in the peripheral lymphoid organs.

Introduction

Immunological tolerance to self-antigen presented in the thymus can be imposed through either negative selection against autoreactive T cells or the induction of T cell anergy. In certain antigen systems the cellular requirements of these two processes have been defined and analyzed at the clonal level. Bone marrow derived cells cause the physical deletion of thymocytes expressing T cell receptors (TCR) specific for self antigens (1–6), whereas thymocytes expressing TCR specific for self antigen expressed exclusively by thymic epithelium are not deleted but are instead rendered unresponsive (anergic) to subsequent stimulation by antigen or by anti-TCR antibodies (7–9). Thus, for the subset of self antigens expressed in the thymus, clonal deletion and the induction of anergy constitute distinct mechanisms for regulating T cell reactivity. However, the mechanism(s) responsible for regulating T cell reactivity to self antigens expressed extrathymically are still being resolved.

Various approaches have been adopted to address the question of how self tolerance is maintained for antigens not expressed in the thymus. Among the most notable of these has been the development of transgenic mouse models (10–19) where genes encoding defined MHC (10–15) or viral (17–19) antigens have been placed under the control of heterologous tissue specific promoters. The consequences of restricted extrathymic antigen expression on *in vivo* and *in vitro* T cell reactivity was then monitored. Although these studies have provided useful insights into the effects of extrathymic antigen expression on the reactivity of T cell populations (reviewed in ref. 20) they generally provided little direct information about the underlying basis for the development of hyporeactivity against the transgene products. In those cases where the integrity of T cell clones specific for the transgene products could be directly assessed, clonal anergy as opposed to clonal deletion appeared to be responsible (14–16).

Using male antigen specific TCR transgenic mice several models were developed to study the regulation of T cell function *in vivo*. The data presented herein are most consistent with a process whereby thymus derived male antigen-reactive CD8$^+$ T cells are specifically eliminated upon encountering male antigen on cells in the peripheral lymphoid organs. Thus, deletion of peripheral CD8$^+$ T cells seems to provide an additional mechanism whereby T cell tolerance to extrathymic self antigen can be maintained.

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Methods

Mice

C57L (H-2^b), C57Bl/6 (H-2^b), and (C57L × bm12)F$_1$ mice were either obtained from Jackson Laboratories (Bar Harbor, ME) or bred in the animal facility in the Department of Microbiology at the University of British Columbia (UBC). Transgenic mice bearing $\alpha\beta$ TCR transgenes on the C57L background were generated as described previously (4). Breeders for this transgenic line were provided by Dr Harald von Boehmer, Basel Institute for Immunology, Basel, Switzerland. Transgenic mice were maintained at the animal facility in the Department of Microbiology, UBC, by backcrossing to C57L mice.

Media

Iscove's modified Dulbecco's medium (IMDM) (Gibco, Burlington, Ontario) was supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml), 5 × 10^{-5} M 2-mercaptoethanol, and 10% fetal calf serum (FCS) (Gibco, lot no. 43N3092).

Antibodies

CD8-FITC [mAb 53-6.7 (21), fluorescein conjugate] and CD4-PE [GK1.5 (22), phycoerythrin (PE) conjugate] were purchased from Becton-Dickinson (Mississauga, Ontario). The transgenic β chain specific mAb F23.1 (23) and the transgenic α chain specific mAb T3.70 (24,25) were biotinylated.

FACS analysis

For fluorescence staining, 5 × 10^5 cells were incubated with the indicated mAb diluted in PBS and 2% FCS for 15 min at 4°C. All staining reagents were used at predetermined saturating concentrations. For analysis of TCR expression, a streptavidin–Tandem (SA-Tandem) conjugate (no. 7100-10, Southern Biotechnology Associates, Birmingham, AL) was used to detect staining with biotinylated TCR specific mAbs. When biotinylated antibodies were used, cells were washed in PBS and 2% FCS once after the 15 min incubation before addition of SA-Tandem. For three-color analysis, cells were incubated with biotinylated mAb specific for either the α or β transgene product, washed twice, incubated with SA-Tandem, washed, incubated with CD4-PE and CD8-FITC, and washed. Cells were finally resuspended in 500 μl of PBS with 2% FCS for analysis with a FACScan flow cytometer equipped with a single argon laser (Becton-Dickinson, Mississauga, Ontario). Dead cells were excluded from data collection by setting a viability gate with forward scatter and side scatter parameters. For two-color analyses 10 000 events were collected per sample. Fifteen thousand events were collected for samples stained for three different markers. Where specified, percentages were determined with FACScan Research software.

Mixed lymphocyte reaction (MLR) and limiting dilution assay

For MLRs, media was also supplemented with IL-2 in the form of supernatants from cultures of IL-2-gene transfected X63/0 cells provided by Fritz Melchers (26). The final concentration of IL-2 used was 20 units/ml, one unit being defined as the dilution of supernatant required to achieve 50% maximal proliferation of the IL-2 dependent CTLL cell line (27) obtained from ATCC (Rockville, MD). Cultures were set up in U-bottom microtitre wells in a 200 μl volume. Varying numbers of responders were combined with 5 × 10^5 irradiated [2000 rad, ^{60}Co source (Gammacell, Atomic Energy of Canada, Ottawa, Ontario, Canada)], C57BL(6 (H-2^b) male, or C57BL/6 female, spleen cells. Between 3 and 4 days after initiation of cultures, all wells received 1 μCi of [^{3}H]thymidine (no. NET-027X, Dupont Canada) diluted in culture media. Between 4 and 6 h later, cultures were harvested onto glass filters, dried, and counted in a scintillation counter. The frequency of T cells that proliferated in response to male antigen was estimated by limiting dilution assay in U-bottom microculture plates. Cultures included 2 × 10^4 male peritoneal exudate cells per well that had been irradiated with 2000 rad, 20 U/ml of IL-2, and graded numbers of responding lymph node cells from the mice specified. Twenty four replicate cultures were set up for each of the responder cell doses used. Nine days later cultures were scored for proliferation using an inverted microscope. Wells containing an estimated excess of 20 viable lymphoblasts were considered positive. Frequency estimates were based upon a linear approximation of data on a semilog non-responder plot as described previously (28).

Apoptosis

DNA fragmentation was assessed as described previously (29). Briefly, 8 × 10^5 cells were pelleted in an eppendorf centrifuge tube and resuspended in 0.4 ml of hypotonic lysis buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 2% Triton X-100; Sigma). Samples were spun for 10 min at 16 000 *g*, and the supernatant was transfered to a new tube. Each tube then received an equal volume of isopropanol and 80 μl of 5 M NaCl. The contents were thoroughly mixed and incubated overnight at −20°C. Samples were then spun for 30 min at 16 000 *g* at 4°C. Precipitates were air dried, resuspended in 5 μl of TE (10 mM Tris, pH 8.0, 1 mM EDTA), 2 μl of loading buffer (0.25% bromophenol blue and 40% sucrose in water), and run on a 1% agarose gel in Tris–borate buffer. As a positive control for DNA fragmentation during apoptotic cell death, DNA was isolated from the same number of thymocytes (8 × 10^6) cultured for 6 h after irradiation with 1000 rad (29).

Results

Male-specific CD8⁺ T cells are deleted in the peripheral lymph nodes but not in the thymus of female transgenic mice neonatally tolerized to the male antigen

In the female TCR transgenic mice used in this study there is an overproduction of mature CD8⁺ thymocytes, the vast majority of which express the $\alpha_T\beta_T$ TCR (where subscript T indicates transgenic) specific for the H-Y antigen plus H-2D^b. This overproduction of transgenic TCR-expressing mature CD8⁺ thymocytes is a consequence of the positive selection of immature thymocytes developing in an H-2^b thymic environment (24,30). The clonally distinct portions of the transgenic TCR are encoded by the V$_\beta$8.2 and V$_\alpha$3 gene segments, the products of which are detected by the F23.1 (23) and T3.70 (24,25) mAbs respectively. As a result of the differential effects of the β and α transgenes on the functional rearrangements of endogenous V$_\beta$ and V$_\alpha$ genes (31,32), and positive selection by the H-2D^b molecule (24,30), the V$_\beta$8.2 and V$_\alpha$3 transgene products are not expressed identically in mature CD4⁺ and CD8⁺ thymocytes. Thus,

mature CD8⁺ thymocytes from female H-2^b transgenic mice are predominantly of the CD4⁻CD8⁺F23.1⁺T3.70⁺ phenotype, and mature CD4⁺ thymocytes from these mice are mostly of the CD4⁺CD8⁻F23.1⁺T3.70⁻ phenotype.

To determine if the development of male specific CD8⁺ T cells in female transgenic mice was affected by induction of neonatal tolerance, female transgenic mice were given an intraperitoneal injection of $2-4 \times 10^7$ male (H-2^b) spleen cells within 24 h of birth. Thymocytes and lymph node cells were collected from uninjected transgenic female mice or neonatally tolerized mice at 6 weeks of age and analyzed as described in Fig. 1. The analysis involved three-color staining of thymocytes or lymph node cells with mAbs directed against CD4 (PE labelled), CD8 (fluorescein-labelled), and either the V_β8.2 transgene (tandem labelled F23.1) or the V_α3 transgene (tandem-labelled T3.70) product. A total of five uninjected and 10 neonatally tolerized female mice were analyzed in this manner and a representative set of data is shown in Fig. 1. In the uninjected transgenic female, mature CD8⁺ thymocytes constituted 26% of the cells and 93% of these expressed exclusively high levels of $\alpha_T\beta_T$ TCR. By comparison, 19% of thymocytes from neonatally tolerized female transgenic mice were of the mature CD8⁺ phenotype and 94% of these thymocytes expressed high levels of the $\alpha_T\beta_T$ TCR. This slight reduction in the percentage of CD4⁻CD8⁺ thymocytes in neonatally tolerized female transgenic mice was a consistent finding (25.6 ± 2.0% SD; $n = 5$ for non-tolerized females versus 19.0 ± 2.0% SD; $n = 10$ for tolerized females). In the lymph nodes of untreated mice CD8⁺ T cells comprised 26%, 68% of which expressed mainly high levels of the $\alpha_T\beta_T$ TCR. The preferential expansion of CD8⁺ T cells of the F23.1⁺T3.70⁻ phenotype in the peripheral lymphoid organs has been noted previously (24). This may be a consequence of priming by environmental antigens. A greater decrease in the percentage of CD8⁺ cells was observed in the lymph nodes of tolerized female transgenic mice (26.5 ± 2.8%; $n = 5$ for non-tolerized females versus 10.6 ± 1.8%; $n = 10$ for tolerized females). More significantly, and unlike the uninjected control, CD8⁺ lymph node cells from the tolerized mice were virtually devoid of cells expressing high levels of the $\alpha_T\beta_T$ TCR.

It can be seen in Fig. 1 that 14% of CD8⁺ T cells in the lymph nodes from tolerized female mice were not stained by the F23.1 mAb whereas virtually all T cells from the lymph nodes of uninjected female mice were F23.1⁺. Similarly, dual staining of tolerized lymph node cells with anti-Thy-1 and F23.1 indicate that 13.0 ± 1.4% ($n = 3$) of Thy-1⁺ cells were F23.1⁻. These Thy-1⁺ F23.1⁻ T cells in tolerized female mice are likely of C57L donor (male) origin since in C57L mice the V_β8 gene family has been deleted and T cells from these mice are consequently not stained by the F23.1 mAb (33). In contrast to the lymph node

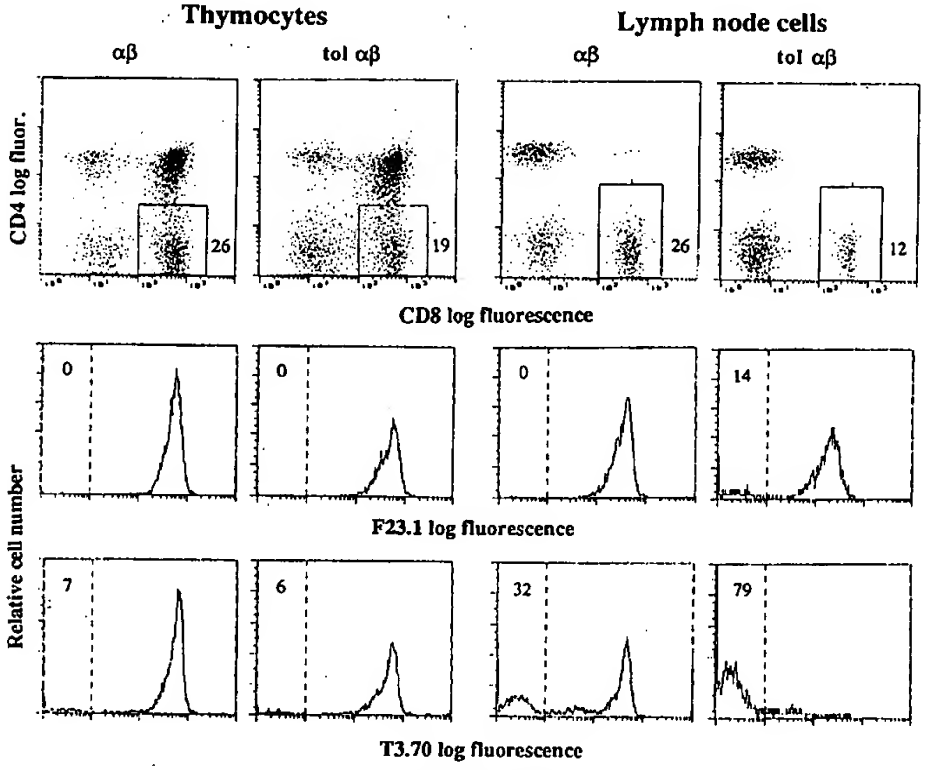

Fig. 1. Male specific CD8⁺ T cells are present in the thymus but not the lymph nodes of αβ female transgenic mice neonatally tolerized with male spleen cells. Thymocytes and lymph node cells from untreated αβ TCR transgenic female mice or αβ TCR transgenic female mice neonatally tolerized to male antigen were isolated and stained with anti-CD4, anti-CD8, and anti-TCR specific mAb as described in Methods. The percentage of CD4⁻CD8⁺ T cells for each population is indicated in CD4 versus CD8 dot plots and the corresponding transgenic TCR staining associated with the CD8⁺ subpopulation indicated is shown in the histograms below (transgenic β chain detected by mAb F23.1 and transgenic α chain detected by mAb T3.70).

Table 1. Deficiency of male-reactive cells in neonatally tolerized lymph node cells

Responder cells	No. of cells/well	Stimulator spleen cells		
		B6 male	B6 female	bm1 female
αβ female thymocytes	3×10^4	78 677	1072	3102
	1×10^4	29 507	537	1776
	3×10^3	10 239	367	1166
αβ female LN cells	3×10^4	56 309	4026	10 499
	1×10^4	14 235	1044	5681
	3×10^3	4643	498	2629
Neonatally tolerized αβ female thymocytes	3×10^4	31 822	644	1242
	1×10^4	8017	437	861
	3×10^3	3077	329	844
Neonatally tolerized αβ female LN cells	3×10^4	6937	2829	8071
	1×10^4	2436	991	3805
	3×10^3	685	420	1983

The indicated number of thymocytes or lymph node cells were cultured with irradiated spleen cells as described in Methods. Each culture was supplemented with 20 U/ml of recombinant IL-2. The cultures were set up in quadruplicate and their proliferate responses assayed at 72 h. [^{3}H]thymidine (1 μCi) was added to each culture during the last 6 h of culture.

cells, all CD8⁺ thymocytes from the tolerized female mice were stained with the F23.1 mAb, indicating their transgenic origin. These data suggest a correlation between donor T cell chimerism and the absence of host derived male-specific CD8⁺ T cells in the lymph nodes of tolerized mice. This deficit in transgenic host-derived CD8⁺ lymph node cells expressing the transgenic TCR was observed at 3 weeks of age and persisted in tolerized females up to 36 weeks after birth, the oldest age at which the mice were analyzed (data not shown). Spleen cells recovered from tolerized animals showed a similar deficit in male-reactive CD8⁺ T cells (data not shown).

We have previously shown that the response by CD8⁺ T cells from female transgenic mice to male stimulator cells correlates with the expression of high levels of the $\alpha_T\beta_T$ TCR (2,34). As illustrated in Table 1, CD8⁺ T cells reactive to the male antigen can be detected in the thymus of neonatally tolerized female transgenic mice despite the paucity of CD8⁺ T cells reactive to the male antigen in the lymph nodes of these animals. Furthermore, the CD8⁺ cells in the lymph nodes of tolerized mice were otherwise functionally normal as assessed by their response to bm1 (an H-2K^b class I MHC mutant mouse strain) stimulator cells (Table 1). Thus, the hyporesponsiveness of CD8⁺ lymph node cells from tolerized females to the male antigen is antigen specific. It should be noted that although the thymocytes from tolerized females responded to the male antigen, this response was consistently about one third that observed for non-tolerized females. One potential explanation for this hyporesponsiveness is that some male cells entered the thymus. Such male cells fail to mediate deletion, as evidenced by the presence of CD4⁺CD8⁺ thymocytes, but may interfere with the maturation/function of mature CD8⁺ male-specific cells as evidenced by their reduced numbers and the greater than expected reduction in the anti-male response. To examine whether the lymph nodes of tolerized mice contained cells which were cytotoxic or suppressive for male-specific CD8⁺ T cells the former were co-cultured with the latter and male stimulator cells. As shown in Table 2 the male-specific response of either thymocytes or lymph node cells from uninjected female mice was unaffected by the presence of lymph node cells from tolerized female mice arguing against the involvement of a cytotoxic or suppressor mechanism regulating the function of male-specific T cells.

The data described above demonstrate the persistence of male reactive CD8⁺ T cells in the thymus but not the lymph nodes of neonatally tolerized mice. These observations contrast with those obtained in studies of neonatally induced tolerance to minor lymphocyte stimulating (Mls) antigens and MHC antigens since in those studies neonatally induced tolerance was found to be associated with the functional deletion of antigen reactive T cells in the thymus (35–41). To determine whether deletion of transgenic TCR-expressing immature thymocytes could occur at earlier times post-tolerization, thymocytes from neonatally tolerized females were analyzed at 8 days following injection of male spleen cells. As shown in Fig. 2, there was no evidence of significant deletion even in 8 day old animals. The proportion of thymocytes expressing low levels of the transgenic TCR in 8 day old animals was equivalent in uninjected mice and female mice injected at birth with male cells. By contrast, the deletion of male-specific precursors in 8 day old male transgenic mice was virtually complete and this deletion was associated with the loss of CD4⁺CD8⁺ thymocytes expressing low levels of the transgenic TCR (2,34). In accord with previous observations, the surviving thymocytes were largely of the CD4⁻CD8⁻ phenotype (Fig. 2) and these cells expressed high levels of the transgenic TCR (2,34). It is also clear from the data in Fig. 2 that even by 8 days of age little positive selection has occurred in the uninjected transgenic mice, this being indicated by the low percentage of mature CD4⁻CD8⁺ thymocytes. By 3 weeks of age, mature CD8⁺ thymocytes expressing $\alpha_T\beta_T$ TCR comprised >10% of thymocytes in female transgenic mice, regardless of whether they were neonatally tolerized with male spleen cells (data not shown). This observation is consistent with our previous

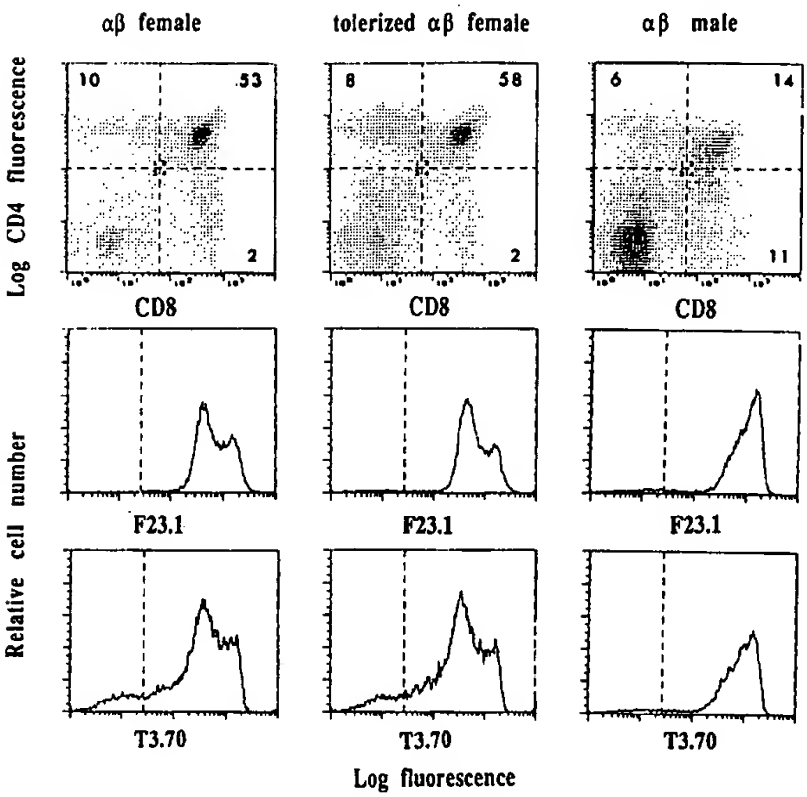

Fig. 2. No evidence of thymic deletion of male antigen-specific cells shortly after injection of neonates with male spleen cells. Eight days after neonatal injection of male spleen cells, thymocytes from αβ female mice were compared with thymocytes from untreated αβ female or αβ male mice for CD4, CD8, and TCR expression. Percentages of the four thymic subpopulations distinguished by CD4/CD8 staining are indicated. The corresponding TCR α-chain specific (T3.70) and β-chain specific (F23.1) staining for the entire thymocyte population is shown below. The biphasic (TCR^{hi} and TCR^{lo}) staining pattern is lost through negative selection against αβ thymocytes in male transgenic mice leaving only αβ TCR^{hi} $CD4^-$ $CD8^-$ T cells whereas neonatally tolerized thymocytes maintain a normal pattern of CD4/CD8 and TCR expression relative to untreated transgenic female control mice.

report that negative selection of $CD4^+CD8^+$ thymocytes expressing this transgenic TCR can precede positive selection by at least 2 weeks (25). This implies that for this particular TCR, positive selection need not precede negative selection.

Preferential loss of male-specific CD8⁺ T cells in the lymph nodes of normal (non-transgenic) female mice neonatally tolerized to the male antigen

One trivial explanation for the lack of male-specific T cells in the lymph nodes of tolerized animals is that male-specific T cells are not exported from the thymus to the lymph nodes of these mice. To determine more directly whether the lymph nodes of tolerized mice were suited for the survival of male specific T cells, 5×10^7 thymocytes from female transgenic mice were injected intravenously into either normal C57L female, normal C57L male, or neonatally tolerized C57L female mice. The composition of injected transgenic thymocytes was: 13% $CD4^+$, 64% $CD4^+CD8^+$, 7% $CD4^-CD8^-$, and 16% $CD8^+$. Three days after injection, lymph nodes of injected mice were harvested. Injected transgenic thymocytes were distinguished from recipient cells using the F23.1 marker which as noted above, is not expressed in C57L mice. To isolate these transgenic cells, lymph node cells were labelled with fluoresceinated F23.1 mAb and sorted using the FACStar Plus cell sorter. The sorted cells were then labelled with (i) mAbs to CD4 (PE labelled) and CD8 (trandem labelled), or (ii) mAbs to CD4 (phycoerythrin labelled) and F23.1 (fluorescein labelled), or (3) mAbs to CD8 (PE labelled) and T3.70 (Tandem labelled). The results of one of three such experiments are illustrated in Fig. 3. Three days after injection about 3% of the lymph node cells were of transgenic origin, staining intensely with the F23.1 mAb. As shown in Fig. 3, fewer $CD8^+$ cells of transgenic origin were recovered from the lymph nodes of the tolerized female mouse (21%) when compared to the non-tolerized female (58%). Consistent with this low recovery of $CD8^+$ cells is the observation that there is a corresponding increase in the proportion of $CD4^+$ cells in the tolerized recipient (54% versus 27%). Nearly all the $CD4^+$ cells were $F23.1^+$ indicating that they were of transgenic origin (compare rows 2 and 3 of Fig. 3). More significantly, there was a 6-fold decrease in the proportion of $CD8^+$ $T3.70^+$ cells in the lymph nodes of the tolerized female mice when compared to the normal control (row 4 of Fig. 3). This preferential loss of male specific $CD8^+$ T cells in the tolerized recipient was even more apparent than that observed in the normal male recipient (Fig. 3).

An independent assessment of the preferential depletion of male-specific cells in the lymph nodes of tolerized female or male mice was provided by determining the frequency of male-specific cells in the lymph nodes of recipient mice injected 3 days previously with transgenic female thymocytes. As shown in Fig. 4, the frequency of male specific T cells in lymph nodes recovered

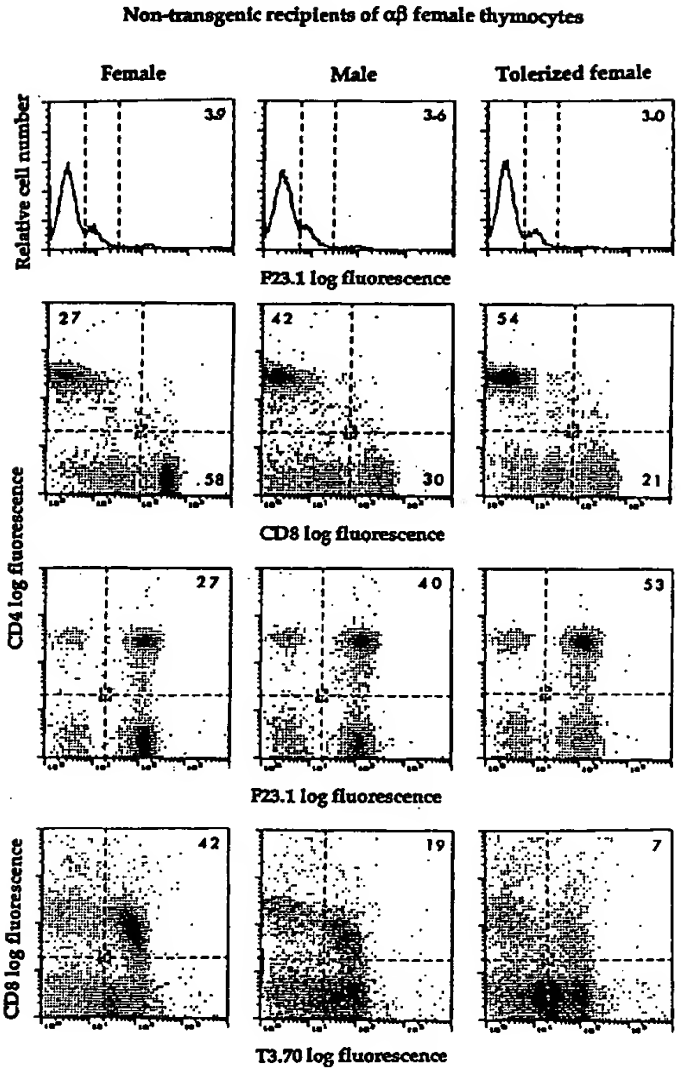

Fig. 3. Selective loss of the CD8⁺ male-specific T cell subset after injection of transgenic thymocytes into non-transgenic male mice or non-transgenic tolerized female mice. Non-transgenic recipients were injected intravenously with 5×10^7 transgenic female thymocytes. Three days later, donor cells were re-isolated from each of the three recipients by cell sorting on the basis of intense staining with F23.1 (top row). The small subpopulation of F23.1⁺ donor (transgenic) origin could be resolved from Fc receptor positive B cells that stained weakly with F23.1 (shoulder on the F23.1 negative peak). The perentage of donor F23.1⁺ cells subjected to cell sorting is given. Sorted cells were then stained for CD4, CD8, or T3.70 expression as described in Methods.

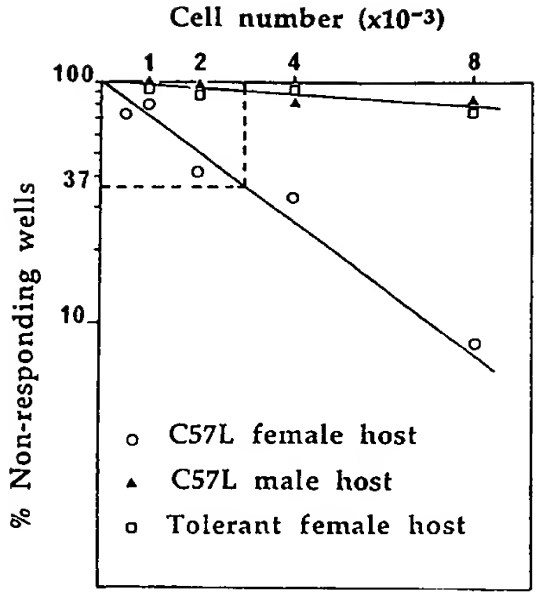

Fig. 4. Clonal elimination of functional male antigen-reactive cells in neonatally tolerant females injected with transgenic female thymocytes. C57L female (O), C57L male (△) or tolerized female (▲) non-transgenic recipients were injected with αβ female thymocytes as described in Fig. 3. Three days later, the frequency of male antigen-reactive cells within lymph nodes was assessed in a limiting dilution assay for proliferative T cell precursors as described in Methods. Data points correspond to the proportion of replicate wells not showing evidence of proliferation at the specified responder cell dose.

from a normal C57L female recipient was 1/3000. By contrast, the frequency of male-responsive cells in lymph nodes of tolerized female or male recipients was 10-fold less, about 1/30 000. The frequency of 1/30 000 represents a background response to IL-2 since this frequency was also observed for all three populations of lymph node cells when they were stimulated with female instead of male stimulator cells. This result suggests that few if any functional male reactive CD8⁺ T3.70⁺ T cells were recovered from the lymph nodes of the tolerant female or male recipient. The staining data in Fig. 3 suggest that some CD8⁺ T3.70⁺ cells were recovered from the lymph nodes of these mice. However, it should be noted that these cells appear to have downregulated their surface expression of CD8. As reported recently, T cell anergy can also be associated with CD8 and TCR downregulation (42,43). Thus, in addition to deletion of male-reactive CD8⁺ T cells, these additional mechanisms may contribute to the lack of anti-male responsiveness.

Evidence for deletion of male-specific CD8⁺ T cells exposed to the male antigen

The experiments described in the two previous sections are consistent with the interpretation that male-specific T cells were deleted upon encountering male cells in the lymph nodes of tolerized animals. However, the possibility that the lack of male-specific T cells in the lymph nodes of tolerized mice may be due to efficient sequestration of these cells in other extrathymic compartments could not be ruled out. Additional data provided more direct evidence that this loss of male-specific CD4⁺CD8⁺ T cells in the lymph nodes was due to specific elimination of male antigen reactive T cells. It was recently reported that staphylococcal enterotoxin B (SEB) reactive $V_\beta 8^+$ splenic T cells underwent programmed cell death (apoptosis) shortly after injection of mice with SEB (44). Cell death was associated with the characteristic fragmentation of genomic DNA (29,45,46). The low recovery of donor female TCR transgenic cells after injection into neonatally tolerant females precluded analysis of apoptosis in these cells. We therefore examined whether there was any evidence of apoptosis of male-specific CD8⁺ T cells in the peripheral lymphoid organs after intravenous injection of female

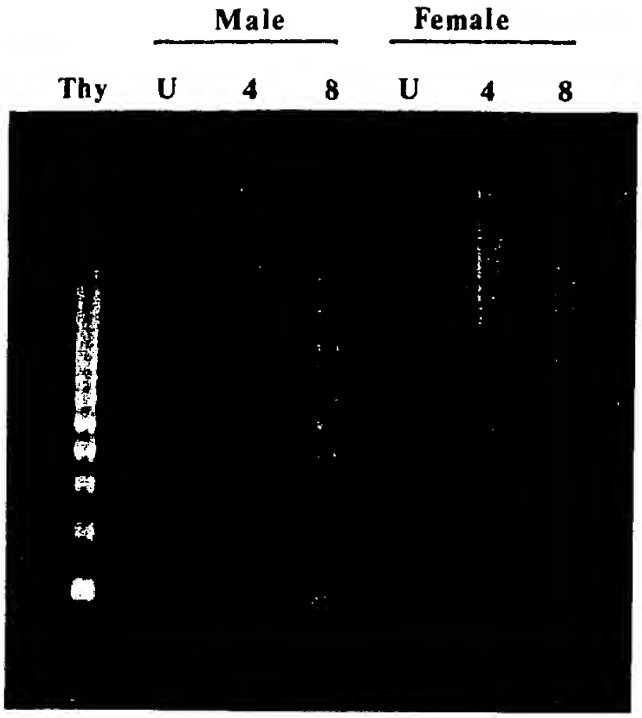

Fig. 5. DNA fragmentation and preferential apoptosis of CD8⁺ cells from transgenic female mice injected with male antigen. DNA was isolated from unseparated (= U) lymph node cells obtained from αβ transgenic female mice injected four days previously with either male or female (control) cells as indicated and compared with DNA isolated from CD4⁺ (= 4) or CD8⁺ (= 8) subpopulations. Irradiated thymocytes (= Thy) provided a positive control for cells undergoing apoptosis and DNA fragmentation (see Methods). The amount of DNA in each lane is the equivalent of the total soluble DNA released by the same number of cells (8×10^5) during the 3 h culture period.

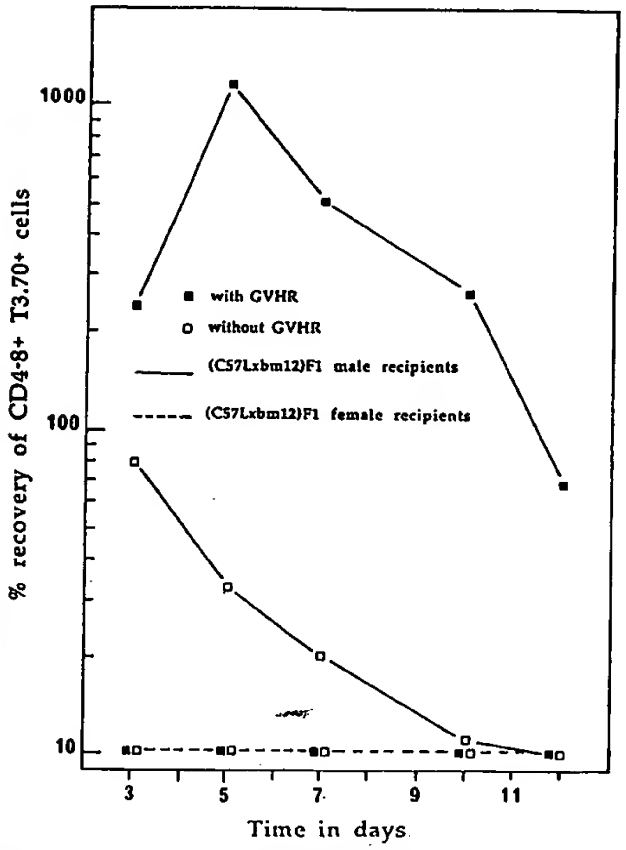

Fig. 6. Prolonged survival and peripheral expansion of CD8⁺ male antigen-specific T-cells in male mice undergoing a GVHR. (C57L × bm12)F_1 female (----) or (C57L × bm12)F_1 male (——) mice were injected with 1×10^7 lymph node cells from αβ transgenic female mice with (■) or without (□) GVHR-inducing C57L female (spleen and lymph node) cells. Spleen cells were isolated from mice at various time points after injection, counted, and stained with T3.70 and anti-CD8 mAb to determine the number of donor derived male antigen-specific T cells. This number was compared to the number of CD8⁺ T3.70⁺ cells present in the 10^7 transgenic T cells originally injected (= 100%).

transgenic mice with 1×10^8 (70% spleen cells and 30% lymph node cells) C57L male or female cells. Four days after injection the lymph node cells from recipient mice were harvested and fractionated into CD4⁺ and CD8⁺ populations by two-color cell sorting. The sorted cells were then cultured for 3 h at 37°C and lysed. Soluble DNA was precipitated and subsequently run on an agarose gel to assess whether the male specific CD8⁺ T cells were undergoing apoptosis preferentially when compared to CD4⁺ T cells which were not male-specific. The results from one of three such experiments are shown in Fig. 5. It is clear that CD8⁺ lymph node T cells from the transgenic mouse injected with male cells had fragmented more DNA when compared to control CD4⁺ T cells isolated from the same mouse and processed in an identical fashion. The background apoptosis by CD4⁺ T cells was comparable to that observed for CD4⁺ and CD8⁺ T cells isolated from female transgenic mice injected with C57L female cells. Such background apoptosis was greater than that observed among unseparated cells, which were kept on ice for the 3 h incubation period, and may therefore reflect non-specific effects from the staining/sorting procedure. It should be noted that the DNA from each lane represented the total soluble DNA released by the same number of cultured CD4⁺ and CD8⁺ T cells during the 3 h incubation period. These data provide direct evidence that male specific CD8⁺ cells can be deleted within peripheral lymphoid organs containing male antigen presenting cells (APCs). However, they do not exclude the possibility that male-specific cells can be sequestered in other extrathymic compartments.

In vivo proliferation of male-specific CD8⁺ T3.70⁺ T cells in the presence of excess activated CD4⁺ T cells

The efficient *in vivo* elimination of male-specific CD8⁺ T cells by male splenic and lymph node cells is somewhat surprising since the same source of APCs can stimulate an anti-male response *in vitro*. Although the basis for this difference remains to be determined it was important to establish that in certain experimental situations the male-specific CD8⁺ T cells could in fact respond to the male antigen *in vivo*. To promote activation and clonal expansion of male antigen specific CD8⁺ T cells upon exposure to male antigen bearing cells *in vivo*, we sought to generate a GVHR in which CD4⁺ T helper cells could be activated by the same cells presenting male antigen to CD8⁺ transgenic T cells. We reasoned that the activated CD4⁺ T helper cells might bind to the same male APC as the CD8⁺

Table 2. Male-reactive cells are not inhibited by lymph node cells from neonatally tolerized mice

Responder cells/well	No. of cells/well	Δ c.p.m. (male – female)	
		Experiment 1	Experiment 2
αβ female thymocytes	3×10^4	36 530	77 605
	1×10^4	12 118	28 970
	3×10^3	4442	9872
Tolerized LN cells	3×10^4	598	4108
	1×10^4	285	1445
	3×10^3	−112	265
αβ female thymocytes + tolerized LN cells (1:1 mixture)	3×10^4	23 026	48 746
	1×10^4	6359	12 979
	3×10^3	1654	4823
αβ female LN cells	3×10^4	48 963	52 283
	1×10^4	15 806	13 191
	3×10^3	4856	4145
αβ female Ln cells + tolerized LN cells (1:1 mixture)	3×10^4	28 131	28 890
	1×10^4	6436	8713
	3×10^3	1905	3232

Culture conditions are as described in Table 1.

male antigen-reactive T cells and the proximation of these two cell types might support the proliferation of male-specific T cells that would otherwise be eliminated. To this end, (C57L × bm12)F_1 male recipient mice were injected with 1×10^7 lymph node cells from female transgenic mice and 1×10^8 C57L female cells (70% spleen cells and 30% lymph node cells). The injected C57L female cells should induce a GVHR against the mutant I-A^b class II MHC molecule expressed by male host cells. Such class II MHC-directed GVHR have been shown to be due to activated CD4⁺ T cells (47). It is clear from the data in Fig. 6 that, in the presence of GVHR-activated CD4⁺ T cells, male-specific CD8⁺ T3.70⁺ T cells proliferated vigorously in the spleens of recipient male mice reaching 10 times the input in the spleen alone, 5 days after injection of the male-specific cells. The proliferation of these cells was antigen specific since only background levels of male specific cells could be recovered from female recipient mice even when they were undergoing a GVHR (Fig. 6). Similar results were observed in the lymph nodes of recipient male mice and at the peak of the male response, 5 times the input cell number were recovered from the lymph nodes (mesenteric, inguinal, auxiliary, and brachial) of male recipient mice undergoing GVHR (data not shown). These data support the conclusion that the efficient deletion of male-specific T cells by male APC *in vivo* can be partially circumvented by an excess of activated CD4⁺ T helper cells. If GVHR-inducing C57L female cells were omitted from the injection, the number of male-specific CD8⁺ T3.70⁺ T cells in the spleen of recipient mice declined rapidly after day 3, falling to background levels by day 11 (Fig. 6). The fact that close-to-input numbers of CD8⁺ T3.70⁺ cells were present in spleens of these mice on day 3, significantly more than the numbers in the spleens of control female recipients, implied that altered trafficking or some degree of clonal expansion was occuring *in vivo*. This proliferation may have been supported by a mild GVHR from transgenic donor CD4⁺ T cells responding to host bm12 alloantigen.

Discussion

In this report, evidence is presented supporting the extrathymic deletion of mature CD8⁺ T cells as one means of achieving peripheral T cell tolerance. Male spleen cells were introduced into female neonates expressing TCR specific for the male antigen presented by H-$2D^b$ class I MHC molecules. The intrathymic, and extrathymic development of male antigen-specific CD8⁺ T cells was subsequently monitored with mAbs specific for the transgenic TCR and by *in vitro* functional assays. The infusion of male cells into neonatal transgenic females prevented the accumulation of male-reactive CD8⁺ T cells in peripheral lymphoid organs. This observation was consistent with previous observations documenting the absence of functional donor-specific host cells in the peripheral lymphoid organs after induction of neonatal tolerance (35 – 41). However, and in contrast to situations where neonatal tolerance was induced to full MHC (35 – 37) or Mls (38 – 41) antigenic disparities, infusion of male cells did not prevent either the development or function of male-specific CD8⁺ T cells in the thymus. We did observe a small but consistent decrease in the proportion of male-specific CD8⁺ T cells in the thymus of neonatally tolerant female mice. This could reflect migration of male donor cells into the thymus in numbers too low to effect complete negative selection. The difference in the degree of central (thymic) deletion of male reactive T cells relative to that reported for MHC or Mls neonatal tolerance systems may also reflect differences in the nature and expression of the antigen in question as well as other phenotypic properties of donor cells gaining access to the thymus. For in-

stance. Webb and Sprent reported that donor Mls^{a+} CD8$^+$ T cells were much more effective than CD4$^+$ T cells and B cells at imposing functional tolerance and intrathymic deletion of Mls^a-reactive host cells (40). They have also reported that different antigens may vary with respect to their capacity to impose tolerance even when expressed on cells of a given phenotype (i.e. thymic epithelium) (48). Given that neonatal tolerance is generally associated with detectable levels of intrathymic chimerism (37) we conclude that male donor cells that enter the thymus are relatively ineffective in inducing intrathymic tolerance induction.

There are only a few reports in which direct evidence of peripheral clonal deletion of mature T cells has been described. Kawabe *et al.* (44) and White *et al.* (49) found that mature SEB reactive, $V_\beta8^+$ CD4$^+$ T cells were eliminated from the thymus (49) and periphery (44) after injecting SEB. The SEB reactive T cells appeared to be eliminated through an apoptotic process (44). Jones *et al.* (50) reported that in IE$^+$ mice treated from birth with anti-IE mAb, $V_\beta6^+$ T cells escaped deletion and accumulated in the periphery. When anti-IE treatment was discontinued numbers of peripheral $V_\beta6^+$ CD4$^+$ T cells diminished progressively. Russell *et al.* (51) reported that mature T cells could be deleted if initially stimulated *in vitro* by APCs in an 11 day mixed lymphocyte culture and subsequently by TCR crosslinking by immobilized anti-TCR antibodies. The relevance of TCR crosslinking by antibody to physiological signalling *in vivo* is unclear but these observations do provide a precedent for the deletion of mature T cells upon TCR engagement. Rocha and von Boehmer (42) found that when male specific TCR transgenic T cells were injected into male nude mice they underwent a transient (5 day) proliferative phase followed by a pronounced decline in cell numbers. Male specific donor cells that persisted after 20 days were no longer responsive to stimulation with male antigen *in vitro*. Finally, Webb *et al.* (52) described similar observations of peripheral elimination of Mls^a-reactive CD4$^+$V$_\beta$6$^+$ T cells injected into Mls^{a+} adult thymectomized mice. CD4$^+$V$_\beta$6$^+$ donor cells entered a proliferative phase after injection into Mls^{a+} mice. Numbers of CD4$^+$V$_\beta$6$^+$ cells reached their maximum numbers on day 4, fell sharply by day 7, and declined to 2% by day 22; those $V_\beta6^+$ cells that persisted were hyporeactive to Mls^a. Although the systems of Rocha and von Boehmer (42) and Webb *et al.* (52) differ in several important respects (male versus Mls antigen, CD8 versus CD4 T cell subset, nude versus adult thymectomized host respectively) the kinetics and pattern of transient proliferation followed by peripheral clonal elimination and hyporeactivity were very similar. In contrast, we found that the kinetics of elimination of CD8$^+$ male specific T cells after injection into normal male mice or neonatally tolerant female mice proceeded much more rapidly. Thus, clonal elimination was nearly complete at day 4 when, in the aforementioned studies, *in vivo* clonal expansion was reaching its peak. Although we cannot rule out the possibility that prior to elimination specific T cells exposed to male antigen undergo a blastogenic response, the tempo of clonal elimination observed makes it difficult to reconcile our observations with a concept of 'exhaustive differentiation' (52) where *in vivo* clonal stimulation and clonal expansion lead to clonal elimination. Both the rate of clonal elimination and the observation that apoptosis of CD8$^+$ T cells was occuring as a consequence of exposure to male antigen *in vivo* seems to be more suggestive of a mechanism of clonal elimination whereby inappropriate signalling has occured in the process of antigen presentation (53).

We have used several systems to reveal extrathymic clonal elimination of male-specific T cells; $\alpha\beta$ female mice rendered neonatally tolerant to male antigen (Fig. 1), male mice injected with $\alpha\beta$ female cells (Fig. 3) and $\alpha\beta$ female transgenic mice injected with male cells (Fig. 5). The fact that male antigen specific cells do not accumulate in significant numbers in the periphery of neonatally tolerant $\alpha\beta$ female mice despite their maturation within, and presumed emigration out of, the thymus suggests that clonal elimination in the periphery of these mice occurs rather quickly. Thus on the basis of specificity (i.e. the requirement for exposure to male antigen expressing cells) and the rapid kinetics of male-specific T cell elimination observed in these three protocols the process of clonal elimination may occur by a common mechanism, i.e. apoptotic cell death. It is quite possible that the increase in apoptosis we observed among sorted CD8$^+$ T cells from female transgenic mice injected with male cells is a significant under-representation of an ongoing *in vivo* process. The lymphocyte isolation procedure selects for viable cells. Under these conditions, the DNA laddering obtained from lymph node cells reflects apoptosis of only that subpopulation of cells that have begun DNA degradation but have not yet developed altered light scattering characteristics associated with the later stages of apoptotic cell death (54). Finally, we have no direct information on the proportion of the 10^8 male cells injected that have recirculated to the lymph nodes and are responsible for inducing apoptosis. The degree of apoptosis observed could therefore be limited by the number of donor male cells that enter the lymph nodes.

The cellular mechanisms responsible for the selective elimination of mature CD8$^+$ male-specific T cells in peripheral lymphoid organs remains to be defined. More specifically, the differences in the response pattern of male-specific CD8$^+$ cells upon exposure to male antigen *in vitro* and *in vivo*, and in normal versus nude male mice, needs further clarification. Nevertheless, several possible explanations for the observed *in vivo* elimination in euthymic male mice can be considered. It is likely that, in the absence of effective priming to the male antigen, the frequency of male antigen specific CD4$^+$ T helper cells in naive populations of T cells from female transgenic mice is too low to provide adequate helper functions required to support a significant anti-male response by CD8$^+$ T3.70$^+$ T cells. Engagement of the CD8$^+$ male specific T cells with cells presenting male antigen may be sufficient to trigger the former to express IL-2 receptors. In the absence of appropriate helper signals the T cell may then be inactivated (55). Accordingly the elimination of CD8$^+$ cells would be a secondary, or passive, outcome of insufficient support from T helper cells. The observation that activated bystander CD4$^+$ T cells can interfere with the elimination of CD8$^+$ cells *in vivo* is consistent with such a hypothesis. However, clonal inactivation resulting from T helper cell insufficiency has most often been associated with the induction of clonal anergy (14–16,55) which is in contrast with the deletion of male specific CD8$^+$ cells observed in this study.

An alternative mechanism that may be responsible for the specific elimination of male antigen specific T cells in the periphery involves the action of veto cells (56–58). The veto model

proposes the existence of two sets of functionally distinct APCs, one set that promotes the activation of resting T cells and a second set that deletes (vetos) precursors of CD4⁺ T helper (59) or CD8⁺ cytolytic (56 – 58) T cells. Here, the male cells injected into neonatal female transgenic mice would contain male-antigen-bearing veto cells that inactivate the transgenic T cells recognizing them. The observation that $\alpha\beta$ transgene bearing cells are deleted in the periphery but not in the thymus (Fig. 1) would imply that veto cells do not accumulate to a significant degree in the thymus. Veto cells can only act on precursor T cells shortly after they have been signalled through their TcR and before they have become mature effector T cells (60). Thus, if stimulatory APC and help are both present in excess, T cells may become activated and receive help before encountering a veto signal. This may explain why transgene-bearing cells can become activated *in vivo*, even in the presence of veto activity, when a very strong helper stimulus is provided (Fig. 6 and ref. 61). The veto model does not provide an explanation for the ultimate elimination of male antigen specific CD8⁺ T cells as shown in Fig. 6, or in male nude mice (42) where T cell activation/proliferation has preceded their elimination. Clearly, either the integrity of CD8⁺ T cells becomes progressively dependent upon support from other cells or additional mechanisms are operating *in vivo* to limit their expansion and survival.

Despite the effectiveness of the peripheral deletion mechanisms involved we could interfere with the elimination of male reactive CD8⁺ T cells by creating a concurrent GVHR directed against alloantigens on host cells co-expressing the male antigen. Under these conditions male (host) antigen presenting cells could theoretically provide a physical link (62, 63) between CD8⁺ male-specific T cells and CD4⁺ T cells activated in the GVHR. It is also possible that *in vivo* proliferation of CD8⁺ male specific T cells during a concurrent GVHR may occur because male (host) antigen presenting cells are effectively converted by CD4⁺ T cells, from a non-stimulatory (deleting ?) phenotype, to a phenotype stimulatory for CD8⁺ T cells (64). In either case the prediction is that the physical potential for simultaneous presentation of antigens on a single presenting cell to both CD4⁺ T helper cells and CD8⁺ T cells would be required to see the effects of GVHR in supporting the proliferation of CD8⁺ T cells *in vivo*.

In summary the study presented provides evidence for the existence of a tolerance mechanism whereby mature CD8⁺ T cells encountering antigen in the periphery can be rapidly eliminated. These results emphasize the potential importance and diversity of extrathymic mechanisms in maintaining T cell tolerance. From the view of organ transplantation in humans, these mechanisms are potentially more relevant than intrathymic mechanisms since they involve the extrathymic tolerization or elimination of mature T cells which are the primary effectors in the rejection reaction. The ease by which alloantigen specific T cell can be deleted or anergized following intravenous injection of allogeneic lymphocytes provides a rationale for transfusion of donor lymphocytes to promote specific tolerance to transplantation antigens. The delineation of the cellular and biochemical mechanisms leading to peripheral T cell deletion or anergy may lead to new strategies for preventing the rejection of transplanted organs in humans.

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Abbreviations

APC	antigen presenting cell
E	endogenous
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GVHR	graft versus host reaction
IMDM	Iscove's modified Dulbecco's medium
MLR	mixed lymphocyte reaction
PE	phycoerythrin
SA	streptavidin
SEB	staphylococcal enterotoxin B
T	transgenic
TCR	T cell receptor

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